JUL 26 1927

THE JOURNAL OF GENERAL PHYSIOLOGY

Founded by Jacques Loeb

EDITORS
W. J. CROZIER JOHN H. NORTHROP
W. J. V. OSTERHOUT

VOLUME X, NO. 6 JULY 20, 1927

PUBLISHED BIMONTHLY
AT MOUNT ROYAL AND GUILFORD AVENUES, BALTIMORE, MD.
BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

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Entered as second-class matter November 25, 1918, at the Post Office at Baltimore, Md., under the act of March 3, 1879.

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*Out of print.

CORRECTION.

On page 675, Vol. X, No. 5, May 20, 1926, fifth line from the top, *75 per cent ether and 25 per cent alcohol* should read *75 per cent alcohol and 25 per cent ether*.

STUDIES ON ENZYME ACTION.

XLV. Lipase Actions of the Whole Trout at Different Ages.

By K. GEORGE FALK, HELEN MILLER NOYES, and I. LORBERBLATT.

(From the Harriman Research Laboratory, The Roosevelt Hospital, New York.)

(Accepted for publication, April 5, 1927.)

INTRODUCTION.

The lipase or ester-hydrolyzing actions of extracts of the whole rat at ages from 3 days before birth until 3 years 15 days, and of extracts of the whole mouse at ages from 6 days before birth until 1 year 261 days were presented in previous communications.[1,2] The changes in the "pictures" of relative actions on ten esters of these extracts as the animals increased in age were given in some detail, and the absolute enzyme actions, which were also recorded, were shown to undergo characteristic changes.

At the suggestion of Dr. C. B. Davenport of the Carnegie Institution of Washington, Station for Experimental Evolution, a similar study was undertaken with trout, because here it was possible to begin with the eggs at a very early stage, and to follow the enzyme actions to the adult fish. The materials were obtained from the State Fish Hatcheries, Cold Spring Harbor, Long Island, N. Y., and the investigation was made possible because of the constant cooperation and helpfulness of Mr. Stanley C. Walters, Superintendent of the Hatcheries.[3]

[1] Falk, K. G., Noyes, H. M., and Sugiura, K., *J. Gen. Physiol.*, 1925–27, viii, 75.

[2] Falk, K. G., and Noyes, H. M., *J. Gen. Physiol.* 1926–27, x, 359.

[3] Thanks are due the State Conservation Commission for permission to use the fish and eggs of the Hatcheries in this investigation, and Dr. C. B. Davenport for the use of laboratory space in the Station for Experimental Evolution where the greater part of the experimental work was done.

Experimental Methods.

Rainbow trout were used in this investigation. In studying the eggs, it was found that different results were obtained for the ester-hydrolyzing actions if whole eggs, finely ground, were used instead of aqueous extracts. It was therefore necessary to study the ground solid materials in a number of cases. In other respects, the experimental methods were essentially the same as those used in the earlier investigations.

The eggs were ground with a weighed amount of sea sand, then either extracted overnight with a definite quantity of water, or portions weighed out to which water was added and the ester-hydrolyzing actions determined directly. The very small fish were treated similarly. The larger fish were passed twice through a meat grinder and then either extracted with water or weighed portions used directly for the enzyme tests. The materials extracted with water were allowed to stand at room temperature overnight, filtered through paper, and portions of the cloudy or turbid filtrates used for the tests. For the experiments with the solids, the requisite amounts of water were added to the different portions and the enzyme tests made directly on these. Toluene was present throughout the extractions and the tests. The mixtures were brought to pH 7.0. The conditions of testing were the same as those described previously; 15 cc. of solution or mixture, 3.4 milli-equivalents of each of the ten esters, 22 hours incubation at 37°–38°, titration with 0.1 normal sodium hydroxide solution with phenolphthalein as indicator; duplicate and blank determinations.

In a number of cases during the summer months, the eggs were removed from the fish after killing the latter. Most of the tests,

---------- Original solids. ————Aqueous extracts.

FIG. 1. Relative lipase actions of trout eggs and whole trout, original solids and aqueous extracts.

Chart *a*. Eggs, 5 hours after being taken. Chart *b*. Eggs, 35 days after being fertilized. Chart *c*. Fish, 2 weeks after being hatched. Chart *d*. Fish, 2 weeks after being fed. Chart *e*. Fish, 3 months after being fed. Chart *f*. Fish, 4 to 5 years old.

The difference in the two curves (original solids and aqueous extracts) are quite marked in Chart *a*, less so in Chart *b*, and still less so in Chart *c*. The two curves practically coincide in each of the last three charts.

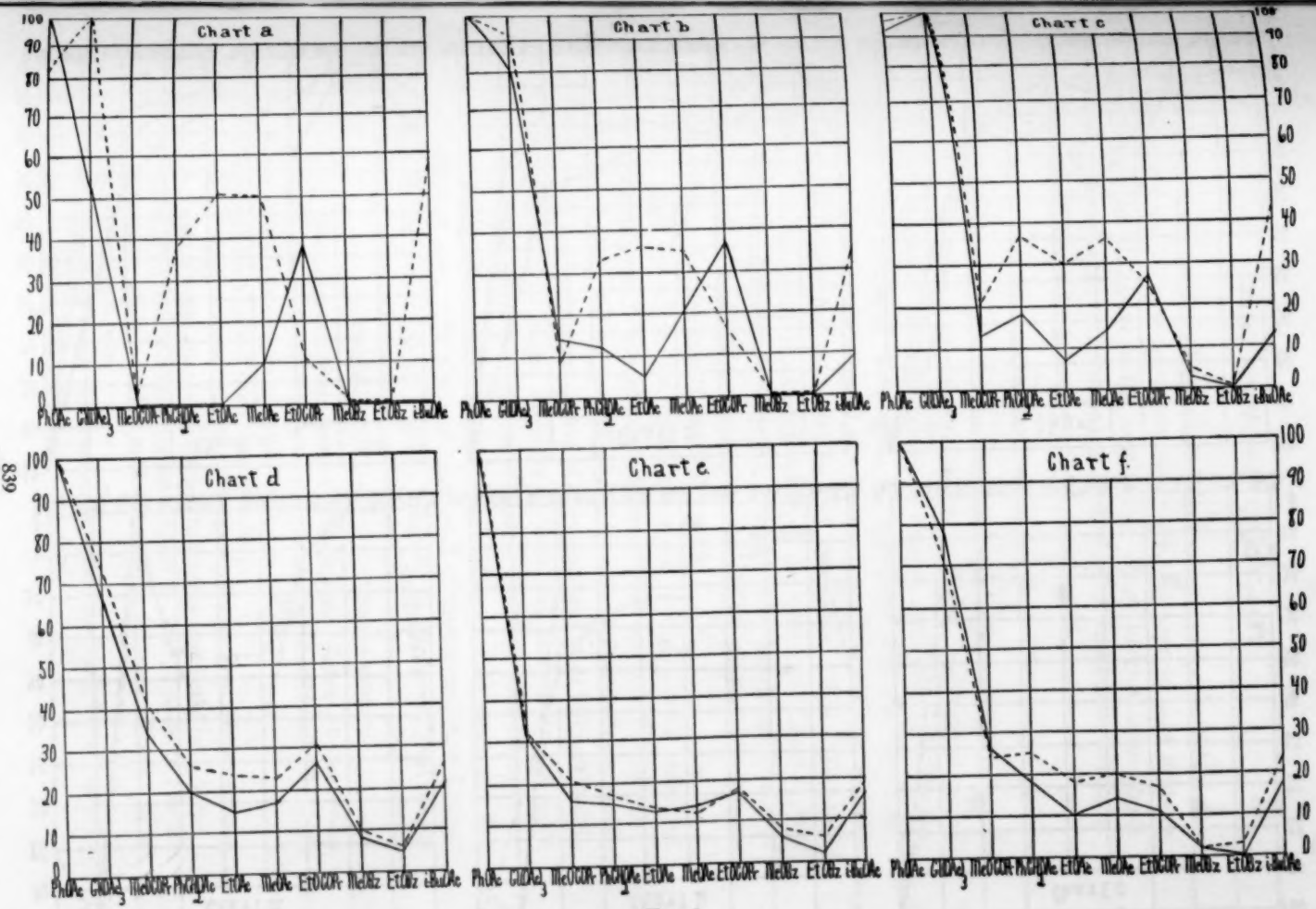


FIG. 1.

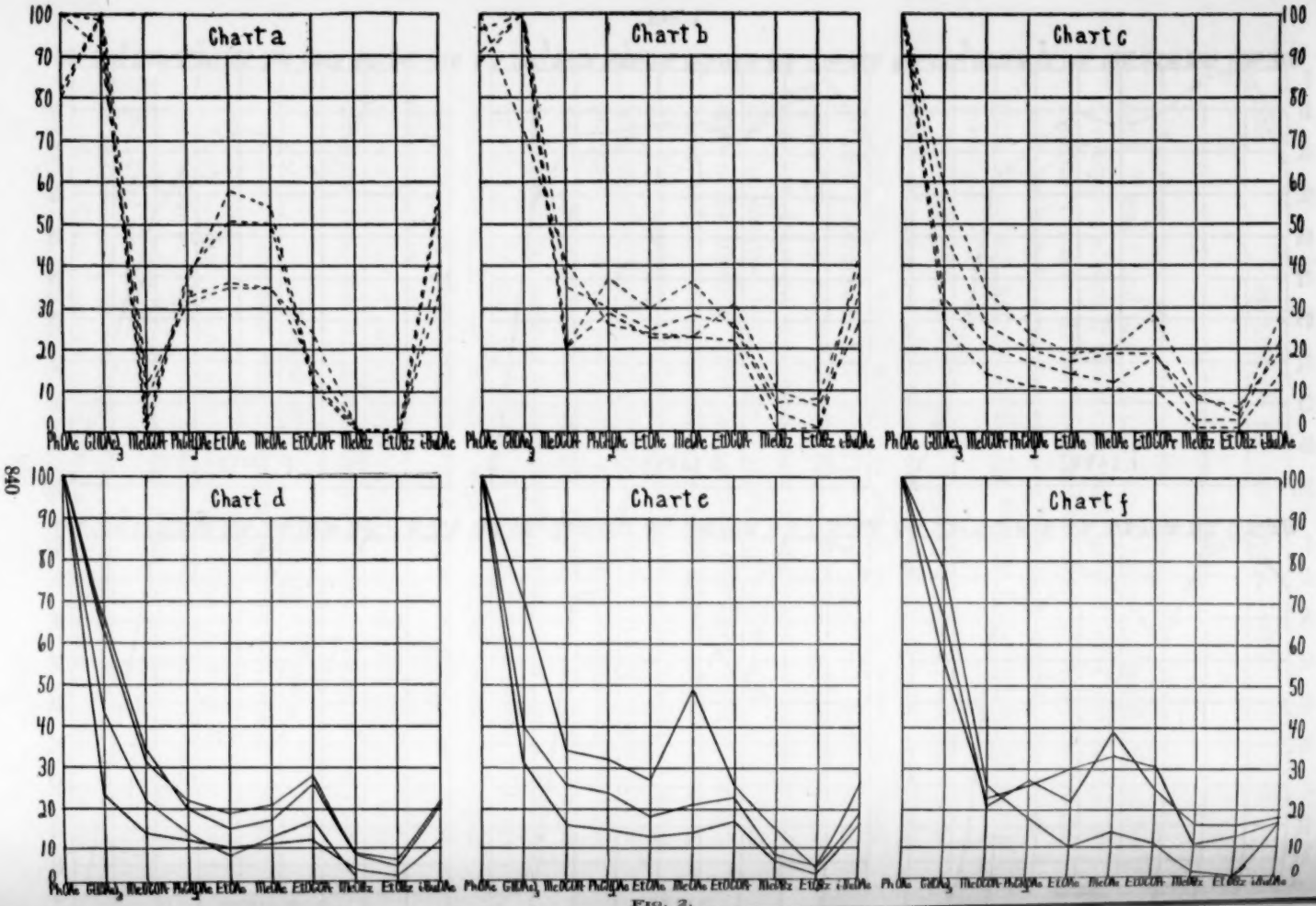


Chart a
Chart b
Chart c
Chart d
Chart e
Chart f
100
90
80
70
60
50
40
30
20
10
0

FIG. 2.

however, were made with eggs taken for hatching purposes in November and later. These eggs were fertilized and kept in cold running water at the Hatchery in the usual way. They hatched after about 50 days, and began eating supplied food about 3 weeks after hatching. The ages of the older fish were only approximated.

EXPERIMENTAL RESULTS.

The relative actions on the ten esters of extracts and of original solids of the same materials are shown in Fig. 1. Six specimens at different stages of the life cycle were chosen, and each chart in the figure refers to one of these ages. It is evident that the "pictures" of the actions for the eggs at the earliest age chosen (Chart *a*) were entirely different for the extracts and the solids. The "pictures" differed considerably from each other at the next age (Chart *b*), but only to a small extent for the fish 2 weeks after hatching (Chart *c*). For the fish 2 weeks after feeding to the oldest studied, the curves were practically identical (Charts *d*, *e*, and *f*).

The eggs, for which the results are given in Fig. 1, were taken for hatching purposes. The absolute actions of the extracts of these eggs, some of which will be presented later, were comparatively small. The extracts of a number of sets of eggs removed after killing the fish, gave considerably larger absolute actions but essentially the same curves as the extracts of the more mature (but less soluble) eggs.

A considerable number of results was obtained with eggs and fish of different ages. In place of giving all of these results, a limited number are presented in Fig. 2. Since it is probable that where

---------- Original solids. ———— Aqueous extracts.

FIG. 2. Trout eggs and whole trout of different ages. Relative lipase actions, each curve representing the ester-hydrolyzing actions of the solid material (Charts *a*, *b*, and *c*) or of the aqueous extract (Charts *d*, *e*, and *f*).

The ages of the eggs and trout were as follows:

Chart *a*. Eggs, 5 hours after being taken to 5 days after being fertilized. Chart *b*. Fish, 2 weeks after being hatched to 2 weeks after being fed. Chart *c*. Fish, 3 weeks to 3 months after being fed. Chart *d*. Fish, 2 weeks to 2 months after being fed. Chart *e*. Fish, aged 3 months to 1¼ years. Chart *f*. Fish, aged 2 years to 4 to 5 years.

The changes in the curves from the earliest eggs to the young fish are clear, but thereafter no regular change could be determined.

TABLE I.

Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced by Aqueous Extracts and Whole Solids of Trout Eggs and Whole Trout of Different Ages on the Indicated Esters.

Extracts	PhOAc	Gl(OAc)$_3$	MeOCOPr	PhCH$_2$OAc	EtOAc	MeOAc	EtOCOPr	MeOBz	EtOBz	*i*-BuOAc
Eggs removed from fish	3.40	2.83	0.18	0.50	0.68	0.80	0.23	0.02	0.00	0.36
Eggs 4 days after fertilization	0.45	0.26	0.00	0.04	0.04	0.09	0.00	0.00	0.00	0.00
Eggs 37 days after fertilization	0.39	0.31	0.02	0.07	0.06	0.09	0.00	0.00	0.00	0.04
Fish, 2 weeks after hatching	0.72	0.78	0.10	0.14	0.05	0.11	0.21	0.02	0.00	0.10
Fish, 1 week after feeding	1.03	1.31	0.28	0.35	0.19	0.26	0.29	0.05	0.05	0.34
Fish, 2 weeks after feeding	3.14	2.08	1.08	0.64	0.48	0.54	0.84	0.26	0.16	0.68
Fish, 3 weeks after feeding	1.66	1.52	0.69	0.43	0.33	0.40	0.64	0.14	0.12	0.46
Fish, 3 weeks after feeding	3.48	1.16	0.66	0.62	0.60	0.62	0.60	—	—	0.56
Fish, 1 month after feeding	3.82	1.64	0.84	0.52	0.30	0.50	0.64	0.06	—	—
Fish, 2 months after feeding	3.62	0.84	0.52	0.45	0.36	0.40	0.43	0.14	0.06	0.44
Fish, 3 months after feeding	2.77	0.85	0.43	0.40	0.36	0.38	0.47	0.16	0.06	0.45
Fish, 6 months old	2.16	0.87	0.56	0.51	0.38	0.46	0.49	0.18	0.11	0.56
Fish, 1½ years old	0.73	0.51	0.26	0.25	0.18	0.45	0.24	0.13	0.05	0.14
Fish, 2 years old	1.21	0.43	0.25	0.30	0.21	0.29	—	0.10	0.14	0.21
Fish, 2½ years old	1.05	0.64	0.20	0.20	0.15	0.25	0.24	0.14	0.06	0.19
Fish, 4 to 5 years old	1.25	0.96	0.32	0.23	0.13	0.18	0.14	0.02	0.00	0.21
Solids										
Eggs, 5 hours after taking	1.66	2.02	0.06	0.76	1.03	1.00	0.23	0.00	0.00	1.17
Eggs, 15 days after fertilization	1.51	1.88	0.01	0.70	1.09	1.02	0.22	0.00	0.00	1.08
Eggs, 50 days after fertilization (just hatching)	2.09	2.07	0.26	0.66	0.74	0.74	0.50	0.00	0.00	0.85
Fish, 2 weeks after hatching	2.97	3.09	0.65	1.13	0.94	1.11	0.78	0.14	0.04	1.34
Fish, 3 weeks after feeding	3.38	2.48	1.39	0.88	0.81	0.77	1.06	0.33	0.20	0.89
Fish, 3 months after feeding	3.95	1.27	0.81	0.67	0.54	0.48	0.71	0.33	0.25	0.77
Fish, 4 to 5 years old	2.52	1.79	0.61	0.62	0.45	0.50	0.42	0.04	0.07	0.60

differences were found in the curves obtained with the solids and the extracts, the significant results are those obtained with the former, the results are shown for the eggs and the youngest fish in the first three charts for the ester-hydrolyzing actions of the solid materials, and in the last three charts for the extracts which are essentially the same as the results for the solids. Chart *c* for the solids and Chart *d* for the extracts refer to the same materials. The changes in the "pictures" of the actions of the youngest eggs up to the time that the young fish began to eat are quite clear. Thereafter the changes are not so distinct although some trends are discernible.

Absolute values of the ester-hydrolyzing actions of a number of the materials are shown in Table I. The results for extracts and whole solids are given for different ages of the eggs and fish. Not all the results which have been obtained are presented, but the data may be considered to be representative of the various actions. The concentrations in all cases refer to 44.4 mg. of original material per cc. of final solution or mixture tested.

Although the results presented in Table I are somewhat irregular, certain definite conclusions may be drawn from them. The extracts of the eggs removed from the fish after killing the latter (before October 1st) gave much larger actions than did the extracts of the eggs taken for hatching (after November 1st). Extracts of these more mature eggs gave only very small actions after fertilization as well as before. After hatching, the actions began to increase, markedly so 2 or 3 weeks after feeding, although some irregularities are apparent at this time, and apparently reached maximum values 1 to 2 months after feeding. For the older fish the actions decreased again.

With the whole solids, the absolute actions increased starting with the eggs attaining comparatively large values for the young fish.

Without going into the details of the absolute actions on all of the individual esters, certain facts of interest may be mentioned. With both extracts and solids, the actions of the eggs on methyl and ethyl butyrates were practically zero. Especially for the solids, the large actions on isobutyl acetate in comparison with the very small actions on ethyl butyrate (its isomer) are striking. After the fish had begun to feed, the actions on the butyrates increased markedly, to decrease again as the fish became older. In general terms, the acetic esters

were hydrolyzed to considerable extents, although here there were considerable variations with the different alcohol radicals. The higher actions on glyceryl triacetate in comparison with those on phenyl acetate for the solids (and to a less extent for the extracts) with the eggs and youngest fish may be indicated. Other regularities might be pointed out but would not add anything of significance to the findings.

DISCUSSION.

In planning this investigation, it was hoped that the methods of enzyme study applied to the life cycles of whole rats and whole mice would be of interest with fish where it would be possible to procure eggs at a very early stage. The experimental study was complicated by the fact that with the eggs, solubility influences played a part, so that the "pictures" or curves of the relative ester-hydrolyzing actions were different for the extracts and whole solids. These differences did not exist with the fish after a short period of feeding. The changes in the "pictures" of enzyme actions from the eggs (solids) to the fish are quite clear, but there appeared to be no further changes of any degree of definiteness as the fish became larger and more mature. After the fish had eaten supplied food for several weeks the type of actions appeared to remain unchanged. Another fact which may have wider significance relates to the comparatively large solubility of the immature eggs in comparison with the insolubility (as far as substances carrying the ester-hydrolyzing actions are concerned) of the more mature eggs taken for purposes of hatching, although the types of the actions were the same for the two.

SUMMARY.

The ester-hydrolyzing or lipase actions of extracts and whole solids of trout eggs and whole trout of different ages were tested on ten simple esters by the method described in previous papers. Differences in solubility of the enzyme materials of the eggs were found. The "pictures" of the relative enzyme actions changed from a type found with immature eggs to a type which became constant for the fish after they had eaten for 2 weeks. After this, the type did not change up to the age of 4 to 5 years (the oldest trout studied). The absolute ester-hydrolyzing actions of the materials were also presented and discussed.

STUDIES ON ENZYME ACTION.

XLVI. The Ester-Hydrolyzing Actions of Whole Trout Preparations under Various Conditions.

By HELEN MILLER NOYES, I. LORBERBLATT, and K. GEORGE FALK.

(*From the Harriman Research Laboratory, The Roosevelt Hospital, New York.*)

(Accepted for publication, April 5, 1927.)

INTRODUCTION.

The ester-hydrolyzing actions of whole trout and trout egg preparations under the conditions of the general investigation in progress in this laboratory offer a comparatively new field of study so that it seemed of interest to determine some of the simpler questions connected with these enzyme actions. In an earlier paper[1] the actions at different temperatures were studied because it was considered possible that the low temperature at which the fish lived might influence the actions of enzymes under different temperature conditions. It was found, however, that the general behavior was no different from that of castor beans, for example, for which the conditions of growth required a comparatively high temperature. In general terms, two main opposing actions were involved in studying the enzyme actions at different temperatures; increase in enzyme action due to rise in temperature, and inactivation of enzyme by heat in the course of the experimental tests. These two factors did not produce the same effects with the actions on the different esters, so that, depending upon the length of time of the action, greater hydrolysis was found at lower temperatures with some of the esters.

In the preceding paper,[2] the ester-hydrolyzing actions of extracts and whole solids of trout eggs and whole trout at different ages were

[1] Noyes, H. M., Lorberblatt, I., and Falk, K. G., *J. Biol. Chem.*, 1926, lxviii, 135.

[2] Falk, K. G., Noyes, H. M., and Lorberblatt, I., *J. Gen. Physiol.*, 1926–27, x, 837.

presented, and the changes in the curves of relative actions and the absolute actions considered.

In this paper,[3] some of the results obtained in the study of the ester-hydrolyzing actions at different hydrogen ion concentrations will be described as well as the actions of extracts obtained with different solvents.

Experimental Methods.

The experimental methods were essentially the same as those described and used in the preceding paper. Adult fish only were used. These were killed, passed twice through a meat chopper, solid portions weighed directly when desired or treated with a definite volume of solvent and toluene added. After extracting overnight the mixtures were filtered through paper, and treated in various ways. For the experiments at different hydrogen ion concentrations, sodium hydroxide or hydrochloric acid was added and the mixtures tested with indicators. For dialysis collodion bags were used. The mixtures or extracts were finally diluted to the proper concentrations, and 15 cc. portions used for the tests on 3.4 milli-equivalents of each of the ten esters in the usual way. Incubation was allowed to proceed for 22 hours at 37°–38° or at 15°–17°, the acid formed being determined with 0.1 normal sodium hydroxide solution with phenolphthalein as indicator. Toluene was present throughout, and duplicates and the usual blanks run.

EXPERIMENTAL RESULTS.

Some results bearing on the ester-hydrolyzing actions at different initial hydrogen ion concentrations are presented in Table I. Experiment F 2 shows that at both temperatures of testing, higher actions were obtained at pH 7.0 than in the more acid solutions. The fact which was brought out in the earlier paper, namely, that some of the actions, especially the action on benzyl acetate, are larger at

[3] The rainbow trout used were obtained through the kindness and courtesy of Mr. Stanley C. Walters of the State Fish Hatchery, Cold Spring Harbor, Long Island, N. Y., by permission of the State Conservation Commission. The writers wish to thank the Commission and Mr. Walters for the aid they have given in making possible the carrying out of this investigation.

the lower temperature if the tests are continued over a sufficient period of time (22 hours in the present instance), is also shown here. The comparatively large concentration of this extract showed the actions at the different hydrogen ion concentrations more clearly than did the results of Experiment F 8 with a more dilute extract. The actions in this experiment were clearly larger at pH 7.0 and 8.0 than at pH 6.0, but there were no clear-cut differences between the actions at pH 7.0 and 8.0.

In view of the extended study made of the lipolytic actions of various tissue and tumor extracts at different hydrogen ion concentra-

TABLE I.

Ester-Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced by Aqueous Trout Extracts in 22 Hours.

	Experiment F 2. Tested 15–17.5° 88.9 mg. per cc.			Experiment F 2. Tested 37.5° 88.9 mg. per cc.			Experiment F 8. Tested 37.5° 44.4 mg. per cc.		
pH	5.0	6.0	7.0	5.0	6.0	7.0	6.0	7.0	8.0
PhOAc	0.75	1.59	2.15	0.87	1.71	2.33	0.54	0.66	0.61
Gl(OAc)$_3$	0.22	0.74	1.20	0.50	0.95	1.22	0.26	0.47	0.59
MeOCOPr	0.15	0.53	0.63	0.39	0.47	0.43	0.21	0.15	0.26
PhCH$_2$OAc	0.17	0.59	1.03	0.36	0.46	0.57	0.19	0.23	0.23
EtOAc	0.08	0.14	0.35	0.17	0.19	0.37	0.09	0.19	0.32
MeOAc	0.00	0.26	0.54	0.18	0.26	0.51	0.06	0.35	0.46
EtOCOPr	0.03	0.16	0.29	0.30	0.30	0.57	0.08	0.17	0.20
MeOBz	0.04	0.08	0.12	0.27	0.10	0.39	0.12	0.12	0.22
EtOBz	0.03	0.04	0.02	0.23	0.07	0.22	0.06	0.17	0.12
i-BuOAc	0.03	0.43	0.71	0.29	0.30	0.51	0.05	0.11	0.21

tions published some years ago,[4] it appeared to be unnecessary to go farther in this direction with the trout extracts, as the results paralleled the earlier findings. It was there pointed out that in the determination of enzyme action at a definite hydrogen ion concentration, two factors operate simultaneously in every case; enzyme action at the given hydrogen ion concentration, and inactivation of enzyme at that hydrogen ion concentration. Also, in the study of lipase actions, the hydrogen ion concentrations of the mixtures may change because of the formation of acid as the action proceeds.

[4] Noyes, H. M., Sugiura, K., and Falk, K. G., *J. Biol. Chem.*, 1923, lv, 653.

TABLE II.

Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced by Various Trout Preparations on the Indicated Ester in 22 Hours.

No. of experiment	Mixture tested	Temperature of tests	PhOAc	Gl(OAc)$_3$	MeOCOPr	PhCH$_2$OAc	EtOAc	MeOAc	EtOCOPr	MeOBz	EtOBz	*i*-BuO Ac
F 9	Water extract	37½°	0.57	0.29	0.17	0.16	0.17	0.16	0.14	0.18	0.01	0.09
	Glycerol extract, *5 per cent*	"	0.61	0.28	0.19	0.20	0.18	0.13	0.15	0.15	0.11	0.15
	" " *20* " "	"	0.91	0.48	0.29	0.23	0.13	0.15	0.17	0.12	0.01	0.22
	" " *50* " "	"	1.01	0.46	0.30	0.28	0.18	0.19	0.23	0.07	0.06	0.16
F 12	Water extract	37½°	0.73	0.51	0.26	0.25	0.18	0.45	0.24	0.13	0.05	0.14
	NaCl " *10 per cent*	"	0.75	0.61	0.13	0.27	0.15	0.25	0.17	0.09	0.25	0.17
	Glycerol extract, *50 per cent*	"	0.92	0.49	0.34	0.22	0.17	0.26	0.18	0.06	0.09	0.20
F 21	Water extract	15°–17°	1.37	0.63	0.43	0.61	0.20	0.27	0.22	0.14	0.10	0.41
	" " (dialyzed vs. running water)	"	0.58	0.18	0.14	0.14	0.08	0.09	0.11	0.07	0.01	0.14
	NaCl " *10 per cent*	"	1.33	1.16	0.74	0.99	0.72	1.02	0.47	0.15	0.11	1.02
	Glycerol extract, *50 per cent*	"	1.47	0.82	0.61	0.58	0.37	0.48	0.37	0.08	0.02	0.47
	" " *50* " " dialyzed vs. distilled water; liquid in bag	"	1.14	0.53	0.41	0.30	0.17	0.23	0.19	0.04	0.01	0.26
	Glycerol extract *50 per cent*, dialyzed vs. distilled water; (outside liquid no action) liquid in bag + outside liquid	"	1.34	0.68	0.51	0.46	0.26	0.38	0.28	0.07	0.03	0.44
	Original solid	"	2.23	1.74	1.08	1.25	0.76	0.86	0.68	0.29	0.19	1.40
	Solid, after dialysis vs. distilled water	"	1.01	0.72	0.26	0.38	0.31	0.27	0.23	0.00	0.00	0.42
	Liquid in bag from solid after dialysis vs. distilled water	"	1.27	0.68	0.40	0.44	0.34	0.31	0.26	0.10	0.06	0.44
	Solid after dialysis vs. distilled water + liquid in bag	"	1.90	1.34	0.80	0.82	0.60	0.68	0.74	0.12	0.10	0.98
	" " " " " " (outside liquid no action alone) + outside liquid	"	1.17	1.01	0.45	0.55	0.50	0.45	0.27	0.10	0.00	0.68

	Outside liquid + liquid in bag from solid dialyzed vs. distilled water	"	1.34	0.84	0.47	0.57	0.32	0.42	0.24	0.05	0.00	0.50
	Solid after dialysis vs. distilled water + liquid in bag + outside liquid	"	2.12	1.51	0.90	0.97	0.64	0.80	0.65	0.13	0.10	1.09
F 22	Original solid	14°	1.61	1.33	0.53	0.63	0.43	0.60	0.37	0.07	0.06	0.60
	Water extract	"	0.86	0.60	0.25	0.27	0.16	0.25	0.14	0.04	0.02	0.28
	Glycerol extract, *50 per cent*	"	0.88	0.74	0.34	0.30	0.21	0.28	0.22	0.03	0.00	0.27
	NaCl " *10* " "	"	0.99	0.91	0.36	0.44	0.39	0.45	0.29	0.05	0.05	0.47
	" " *10* " " dialyzed vs. distilled water, liquid in bag	"	0.76	0.71	0.28	0.21	0.16	0.21	0.15	0.00	0.00	0.22
	NaCl extract, *10 per cent*, dialyzed vs. distilled water (outside liquid no action alone) liquid in bag + outside liquid	"	1.06	0.93	0.36	0.42	0.33	0.43	0.25	0.02	0.00	0.41

"The difficulty of an exact interpretation of the most favorable hydrogen ion concentration for such lipase actions is therefore apparent. The study of the actions at different hydrogen ion concentrations, definite at the commencement of the actions, involves changes in the reactions and therefore gives results under changed, or continuously changing, conditions. The study of the rates of inactivation by testing at the same hydrogen ion concentration, after permitting the mixtures to stand at different hydrogen ion concentrations, yields definite conclusions, but really involves a problem essentially different from that of actions at definite hydrogen ion concentrations. . . . . It is, of course, possible that the comparative rates of action and of inactivation of certain lipase preparations may be such that definite optima are obtained at certain hydrogen ion concentrations. In the experiments described here,[4] it is seen that the greater actions in the more alkaline solutions more than make up for the inactivations in these solutions, while in the more acid solutions, the reverse is the case."

In view of the results with trout extracts and the general conclusions just stated, it may suffice to state that favorable conditions for testing the trout extracts would be in the neighborhood of pH 7.0, that the meaning of optimum hydrogen ion concentration for the actions must be more sharply defined to have a real significance and may even then serve no practical purpose. In general terms, similar relations apply to the work with trout as with the other tissues and tumors studied. Further results will therefore not be communicated in this connection.

A large number of experiments was carried out in connection with various solvents for extraction and the behaviors of the extracts on dialysis, etc. The four experiments for which the results are given in Table II may be looked upon as representative of the general findings.

The trout for which results are given in Table II were $1\frac{1}{2}$ to $2\frac{1}{2}$ years old. The experiments were carried on at pH 6.0 for F 9, and at pH 7.0 for the rest. The concentrations for the enzyme materials for these experiments corresponded to 44.4 mg. of original material per cc. of solution tested. In the preparation of the extracts, amounts of ground fish were weighed out and liquid added to correspond to three times this concentration. This permitted increases in volume in dialysis and still allowed the final concentrations to be the same. In every case, therefore, before the enzyme tests were carried out, the extracts were made up to three times their original volumes with water. This meant that where salt or glycerine solutions were used for the extractions, their concentrations were reduced to one-third

in the enzyme tests. Thus, where the extract was stated to be made with a 50 per cent glycerol solution, the enzyme tests were carried out with a $16\frac{2}{3}$ per cent glycerol solution, etc.

The results of the four experiments as far as use of different solvents was concerned, showed that glycerol solutions, especially the higher concentrations, extracted more of the active enzyme material than did water alone. The differences, while not large, were still appreciable. There were no such differences with the sodium chloride solution extracts, the enzyme activities, at times slightly larger, were in general much the same as for the aqueous extracts. The results for the more dilute sodium chloride solutions, while not given, were essentially the same as for the 10 per cent solutions. To sum up, it may be said that the glycerol and sodium chloride solutions extracted at least as much of the active enzyme material from the fish as did the water, and in some cases extracted more.

In the dialysis experiments, it was found possible to separate out material, inactive in itself, while the material from which it was separated showed decreased activity. Combining the two, the original activity was restored. In other words, a so called coenzyme was shown to exist, analogous to the results with eel preparations.[5] In the present connection this was shown to be present in Experiment F 21 with the 50 per cent glycerol extract, in Experiment F 22 with the 10 per cent NaCl extract. There were indications of the same relations with the solid material of Experiment F 21 for some of the ester actions, but the differences were not sufficiently large or uniform to warrant definite conclusions.

SUMMARY.

The ester-hydrolyzing actions of trout extracts at different hydrogen ion concentrations were studied. It was pointed out that the behavior was similar to that found with various tissue and tumor materials and that similar relations hold.

Glycerol solutions extracted somewhat larger amounts of active enzyme material from trout than did water and sodium chloride solutions, although the differences were not large. Evidence was presented for the existence of a so called "coenzyme."

[5] Noyes, H. M., Lorberblatt, I., and Falk, K. G., *J. Gen. Physiol.*, 1926–27, x, 1.

THE ANALYSIS OF THE DIVISION RATES OF CILIATES.

By OSCAR W. RICHARDS and J. A. DAWSON.

(From the Laboratory of General Physiology and the Zoological Laboratory, Harvard University, Cambridge.)

(Accepted for publication, April 11, 1927.)

The existence of data on the division rates of representatives of three orders of ciliate protozoans grown in pedigree isolation culture with the same culture medium for 3 years (Dawson)[1] invites analysis to determine the influences which affect the division rates and the relations between the respective division rates.

The division rates of *Paramecium aurelia* (mutant), *Blepharisma undulans*, and *Histrio complanatus* were determined by Dawson. The fact that the culture conditions were kept the same for all three organisms, at any given time, insures homogeneous data.

I.

The changes in the division rate of unicellular organisms are probably best indicated by a staggered or running average; a convenient time interval is 10 days. Averages of the 10 day daily rates made at the end of 10 day periods show the same changes, but with less objective clearness and accuracy. Running averages and the corresponding 10 day averages, for a few days, are plotted in Fig. 1 to show the relations between them. The histogram plot, commonly used by protozoologists, implies that the average rate was maintained constant for the entire 10 day period. Consequently, the histogram is not only theoretically incorrect but may be misleading practically in the interpretation of data.

The data analyzed in this paper are those of 10 day division rates per animal, per day, for each species, for the period between January 30, 1924, and January 14, 1927; with the exception that the *Paramecia*

[1] These observations have been published in part, and the rest will be published later. Dawson, J. A., 1926, *J. Exp. Zool.* xliv, 133; 1926–27, xlvi, 345.

rates started on July 18, 1924, and that the *Histrio* line died on Feb. 8, 1926.[1] The rates for these organisms are separately plotted in Fig. 2, *a*, *b*, *c*.

II.

Inspection of the curves representing the division rates for these organisms shows that there are distinct secular trends for each curve and that each trend has a different slope. It is therefore necessary to

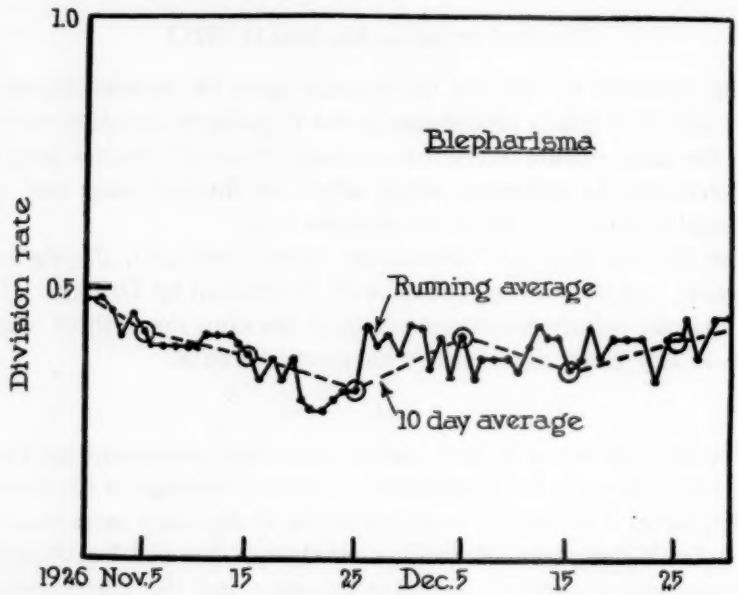


FIG. 1. The comparison between 10 day and running averages.

remove these trends before we can compare the division rates. The ordinates of a line of trend are determined for the beginning, center, and the end of the curve and the line is drawn through these ordinates of trend.[2] The resulting trend is then removed graphically with the aid of dividers. Two sources of error are possible with this method. One is an error made in measurement with the dividers. Since the scale of the original graphs was chosen so that the distance between units was 5 mm., an error of this magnitude is not probable with care-

[2] Rietz, H. L., Handbook of mathematical statistics, New York, 1924, 159.

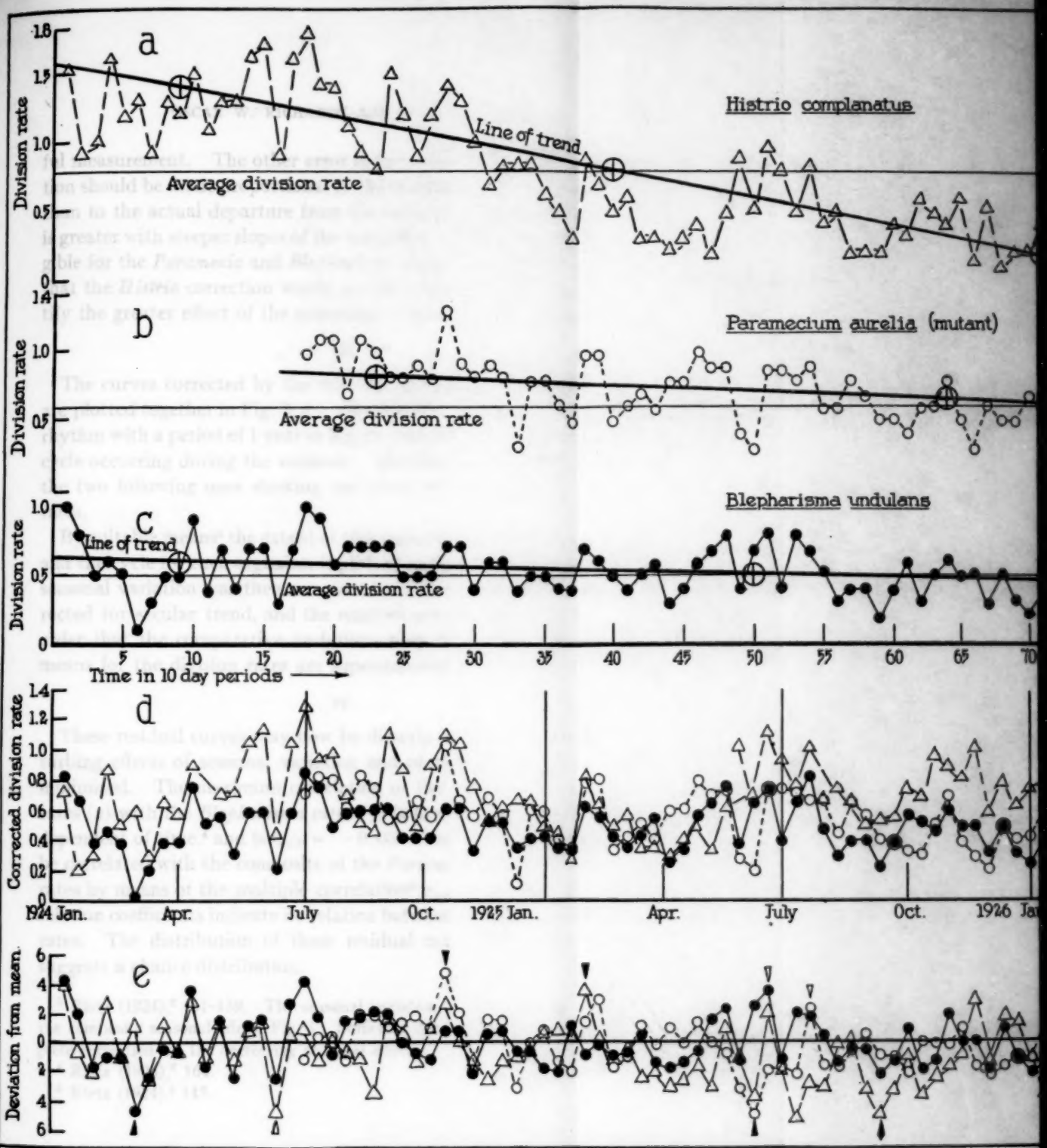
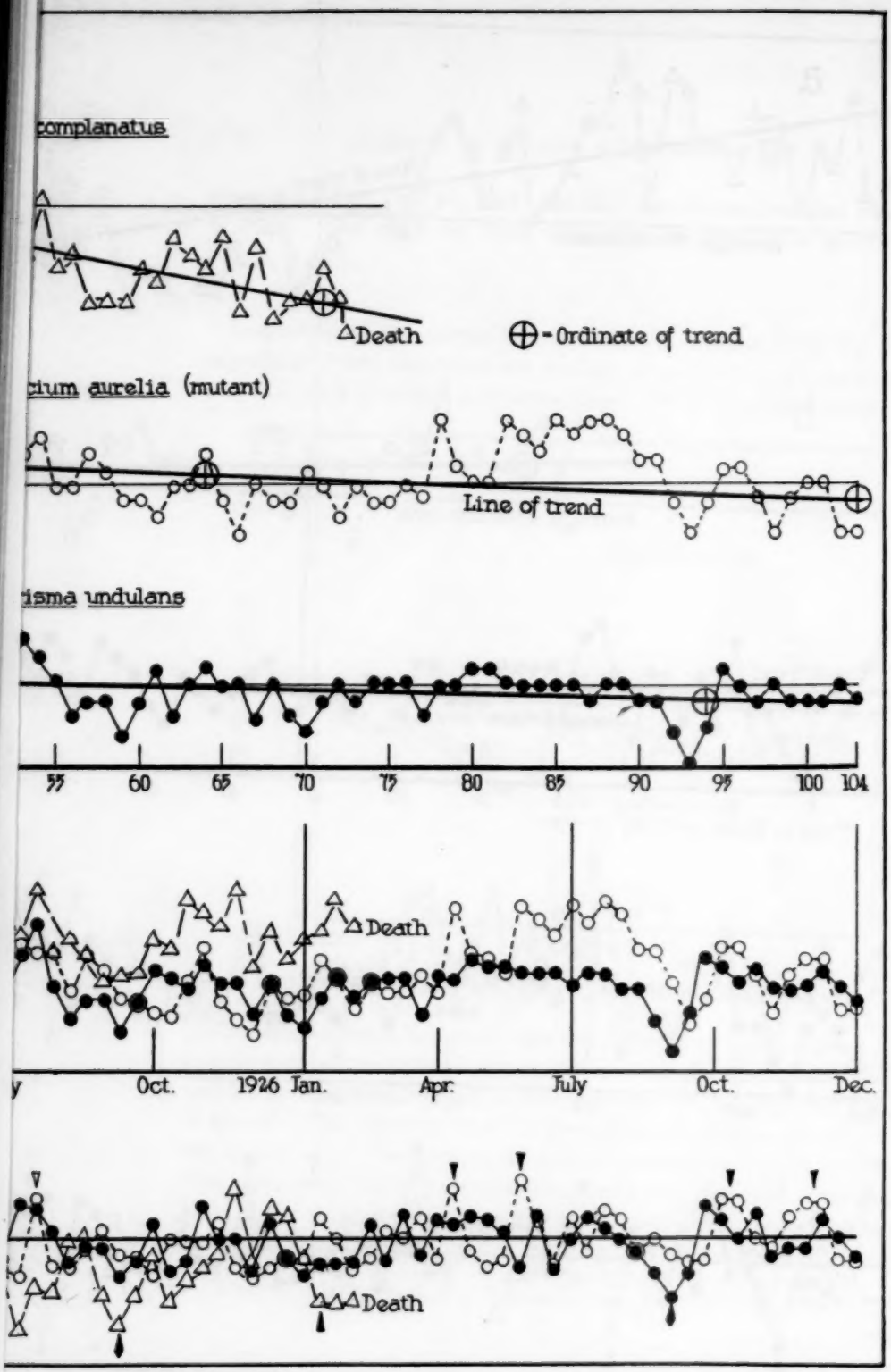


FIG. 2. *a.* Average division rates and line of secular trend of *Histrio complanatus*. *b.* Average division rates and line of secular trend of *P* trend of *Blepharisma undulans*. *d.* Average division rates corrected for secular trend. *e.* Division rate residues after minimizing secular tr △ *Histrio complanatus*. ○ *Paramecium aurelia*. ● *Blepharisma undulans*. ▼ Deviations associated with a change in water sou Woods Hole or to Cambridge. ◆ Deviations associated with temporary change of technician during the absence of Dawson.



rates and line of secular trend of *Paramecium aurelia*. *c*. Average division rates and line of secular esidues after minimizing secular trend and seasonal variation.

ıted with a change in water source. ▲ Same as ▼. ▽ Deviations associated with removal to e absence of Dawson.

ful measurement. The other error is due to the fact that the correction should be made proportional to the magnitude of the trend rather than to the actual departure from the ordinate of trend. This error is greater with steeper slopes of the trend line. The correction is negligible for the *Paramecia* and *Blepharisma* data and calculation showed that the *Histrio* correction would not be sufficiently improved to justify the greater effort of the numerical over the graphical correction.

III.

The curves corrected by the removal of the effect of secular trend are plotted together in Fig. 2, *d*. The division rates now show a cyclic rhythm with a period of 1 year in length, and with the maximum of the cycle occurring during the summer. The first cycle is most distinct, the two following ones showing less effect of season on the division rates.

By suitable means[3] the extent of this seasonal cycle was determined, and the cycle for each organism is plotted in Fig. 3. The effect of the seasonal variation was then removed from the curves previously corrected for secular trend, and the residues are shown in Fig. 2, *e*. In order that the comparative variations may be clearly indicated the means for the division rates are superimposed on this graph.

IV.

These residual curves may now be directly compared since the disturbing effects of seasonal variation and of secular trend have been minimized. The maximum correlation of the corrected *Paramecium* rates (x) with the *Blepharisma* rates (y) is their partial correlation independent of time,[4] and is $r_{xy \cdot t} = -0.08$. The *Histrio* rates (z) may be correlated with the composite of the *Paramecium* and *Blepharisma* rates by means of the multiple correlation[5] $r_{z \cdot xy} = 0.29$. These correlation coefficients indicate no relation between the corrected division rates. The distribution of these residual rates about their means suggests a chance distribution.

[3] Rietz (1924),[2] 151–159. The seasonal variation is minimized by means of the composite seasonal effect, Fig. 3. Sufficient data are not available for complete correction for the decreasing seasonal effect.

[4] Rietz (1924),[2] 164.

[5] Rietz (1924),[2] 145.

V.

If all conditions influencing the division of the protozoans could be kept constant we should expect the organisms to divide regularly and the probability of occurrence of division would be constant. By means of the Lexian ratio we may test the "corrected division rate data" to see if the divisions occur with a constant probability. The empirical probability of a division may be calculated from the average division rate and the sum of the divisions. From this information it is possible to determine the numerical value for the relative Bernoulli

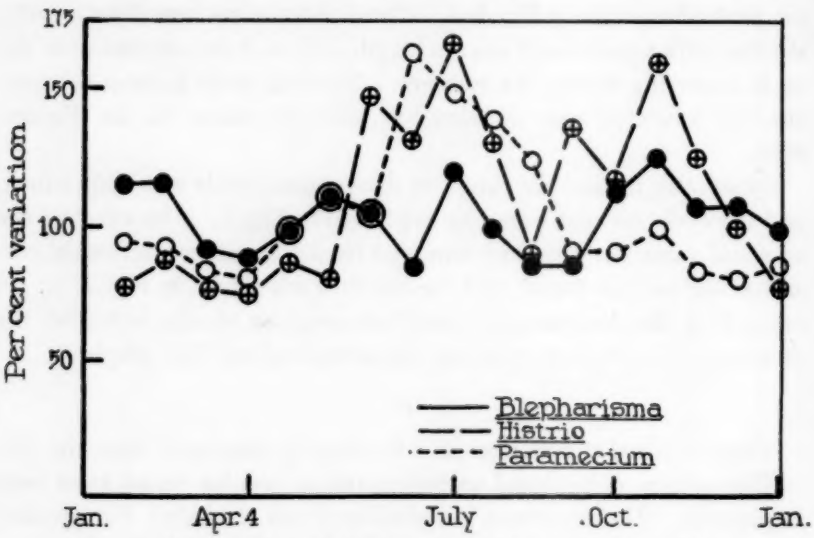


FIG. 3. The yearly cycle of seasonal variation for each organism.

standard deviation for our series; then the Lexian ratio is the ratio of the relative standard deviation of the series to the relative Bernoulli standard deviation for the same series.

The numerical values for the Lexian ratio are given for each organism in Table I for the original data, for the corrected data, and for the corrected data omitting those observations known to be caused either by a change of medium, or by removal from Cambridge to Woods Hole, or return, or by changes in culture technique.

Purely chance deviations of a constant probability would give a Lexian ratio close to unity. The deviations between the calculated

ratios and unity are probably due to variations in technique and in culture conditions that we cannot completely describe at present.

VI.

The analysis shows that after the effect of long secular trend is removed the influence which causes similar variations in the division rates of these three ciliates of diverse orders is a seasonal one. The period of this rhythm is yearly and the maximum of the effect occurs in July. The effect of this rhythm remains distinct although it is disturbed by the annual moves to and from Woods Hole at this time. The effect of the rhythm becomes less the longer the organism is maintained under laboratory conditions. The 1st years of Woodruff's 8 year culture[6] and of Metalnikow's 10 year culture[7] show seasonal

TABLE I.

The Lexian Ratios of the Division Rate Residues after Correction for Secular Trend and Seasonal Variation.

Organism	Original data	Corrected data	Corrected data less known disturbances
Paramecium aurelia	2.19	1.54	1.19
Blepharisma undulans	1.81	1.61	1.31
Histrio complanatus	3.28	1.50	1.21

rhythms that diminish under laboratory conditions. The mechanism of this diminished seasonal effect could be determined under known conditions, and it would be of interest to do so.

These same series also show definite secular trends. The trend in Woodruff's *Paramecium aurelia* is upward and distinct. That of Metalnikow's *Paramecium caudatum* is also upward but with less slope. This analysis of our own division data leaves no trace of cycles or rhythms that might be attributed to recurring cellular reorganization.[8] The four "cycles" from Calkins that Wilson[9] publishes may be ex-

[6] Woodruff, L. L., 1921, *Proc. Nat. Acad. Sc.*, vii, 41.

[7] Metalnikow, S., 1922, *Compt. rend. Acad.*, clxxv, 776.

[8] A forthcoming paper by Dawson will contain a further discussion of this problem.

[9] Wilson, E. B., The cell in development and inheritance, New York, 3rd edition, 1925, 241.

plained by noting that the first two are due to a change to beef extract culture medium; and that the third and fourth, after they are corrected for the steep trend, show one yearly, seasonal cycle very similar to those of our own data and to those of the 1st years of Woodruff's and of Metalnikow's curves.

VII.

SUMMARY.

1. Analysis of the division rates of *Paramecium aurelia* (mutant), *Blepharisma undulans*, and *Histrio complanatus* grown separately in pedigree isolation culture with the same culture medium, and in the same room at any given time, for a period of 3 years, discloses a secular trend and a seasonal rhythm for each organism. The seasonal rhythm is a yearly cycle with a maximum during July.

2. After removal of the effects of trend and seasonal rhythm, no correlation is found between the division rates of the several organisms. The distribution of the division rates is then one of chance order, except for large deviations known to be associated with changes in the culture technique. Each organism has a division rate varying independently of the others.

3. Consequently, seasonal rhythm alone has forced similar variations in the division rates of these three protozoans. The seasonal effect is gradually lost when the animals are raised for several years under laboratory conditions. Examination of the literature discloses other similar cases.

4. It is clear that unless all of the conditions of experiment are kept *constant*, one must analyze all protozoan division rate data in some such manner as that here presented before any conclusions may be drawn as to the existence of "cycles" or "rhythms."

STUDIES ON THE AMOUNT OF LIGHT EMITTED BY MIXTURES OF CYPRIDINA LUCIFERIN AND LUCIFERASE.

By KENNETH P. STEVENS.

(*From the Physiological Laboratory, Princeton University, Princeton.*)

(Accepted for publication, May 3, 1927.)

INTRODUCTION.

Interest has centered for a long time on the intensity and efficiency of animal luminescence, not only from a theoretical but also from a practical standpoint. With the exception of the beetles, most luminous animals emit light in such small amounts that a dark-adapted eye is required to register their intensities. It is a difficult matter to measure accurately low light intensities, especially when they are intermittent as in certain luminous organisms. The intensities of *Cypridina*, however, are sufficiently bright to measure.

Harvey, 1925, has successfully measured the amount of light and over-all luminous efficiency of dense emulsions of luminous bacteria, and finds that they are 0.156 per cent efficient. As is well known the light production in *Cypridina* is an oxidation process, due to the oxidation of a substrate, luciferin, in the presence of a catalyst, luciferase, and follows the laws of enzyme action. After mixing luciferin and luciferase solutions a gradual decrease of luminous intensity follows the initial marked brilliancy. Studies of these decay curves by Amberson by a photographic method show that the light intensities are continually and gradually falling off. In general the effect of the enzyme, luciferase, is proportional to its concentration. Amberson's curves show that the reaction velocities follow this law, *i.e.* the velocity constant is proportional to the concentration of the enzyme. Amberson assumed after Trautz that, ". . . . if light intensity at any instant is assumed to be proportional to reaction velocity at that instant, the decay curve of the light produced in the course of the luminescent reaction in *Cypridina* is, after the first second, in com-

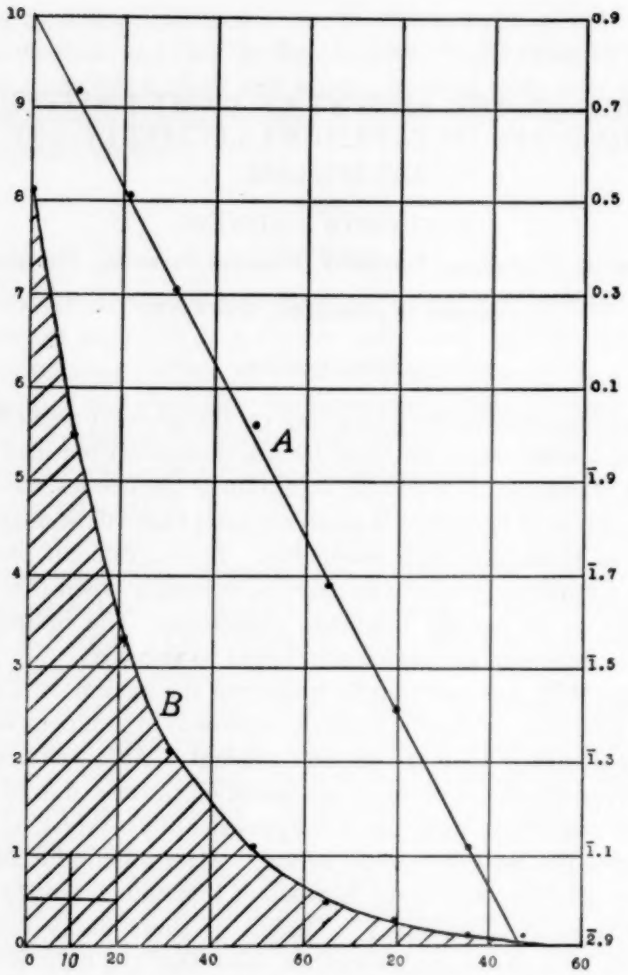


FIG. 1. Typical curve of *Cypridina* luminescence. *A*, logarithmic plotting; *B*, ordinary plotting of luminescence intensity against time. Ordinates, right, logarithms of intensity; ordinates, left, intensity in arbitrary units. Abscissæ represent time in seconds.

plete agreement with the theoretical expectation for a monomolecular reaction." In other words the rate of change of the concentration of *A* at any instant is proportional to its concentration at that instant, or

$$dx/dt\alpha(a-x)$$

where a is the number of molecules of A present originally, and x is the number of molecules of A changed in t minutes; $(a-x)$ is the concentration of A after t minutes or

$$dx/dt = k_1 (a-x)$$

where k_1 is the velocity constant. k_1 has a characteristic value for such reactions and is a measure of the rate of reaction. By integration one obtains

$$k = 1/t \ ln \frac{a}{a - x}.$$

When the intensities are plotted against time, a typical decay curve is obtained (Fig. 1), and when the logarithms of intensities are plotted against time, a straight line is obtained. Since the reaction velocities are proportional to the enzyme concentration, the velocity constants should vary with the luciferase concentrations, but not with the luciferin concentrations. Logarithmic plottings of different luciferin concentrations are parallel, provided the luciferase is present in the same concentrations, whereas the logarithmic plottings of different luciferase concentrations have different slopes.

The present paper records experiments carried out with *Cypridina* luciferin and luciferase in an attempt to measure the total amount of light emitted with various concentrations of the substrate and enzyme. The photographic method of Amberson was adequate for measuring the intensities in arbitrary units and the reaction velocities, but is unadaptable for determining the total amount of light emitted by a unit volume of luminous material in lumens.

A problem concerned with the dynamics of bioluminescence is beset with the difficulty of dealing with a very complex system. All attempts to purify either the enzyme or the substrate are unsuccessful. Furthermore, to obtain the luminous substances, the whole dried body of the organism must be extracted. Hence one is trying to measure one reaction, resulting in luminous intensity, in a system which may exhibit secondary oxidations, or additional modifying factors. In the following experiments anomalous and inexplicable factors control certain features of the reaction.

Materials and Methods.

Cypridina hilgendorfii Müller, the Japanese ostracod, was the organism which was used for the source of light in all the experiments. The desiccated animals were powdered and kept over $CaCl_2$. Great variations occur in the brilliancy of the luminescence from such powder depending upon the care with which the animals were originally gathered and dried, as well as the methods employed in preparing the extracts. It is necessary, therefore, to have the best available stock for studies upon luminescence.

The aqueous solutions of both the enzyme, luciferase, and the substrate, luciferin, have been freshly prepared for each series of experiments. In this way the minimum deterioration of solutions has been obtained. In no case has the enzyme been used after 6 hours from the time of preparation. Luciferase has been prepared in the standard manner by extracting the dried powder with cold sea water, or ordinary distilled water, and allowing the solution to stand until the contained luciferin has been completely oxidized to oxyluciferin. Such a solution is entirely non-luminous to a dark-adapted eye. The solution is then filtered and kept as the stock enzyme solution. Three series of experiments were completed with a distilled water extract; in all others sea water extracts were used. In all cases 0.25 gm. of powder to 50 cc. water have been used in preparing the luciferase.

The luciferin solutions were prepared in two different ways. Ordinarily 1 gm. of dried powder was extracted with 100 cc. N/10 HCl acid sea water. The *Cypridina* powder was added to the sea water at the moment of boiling and the boiling continued for a few moments in order to completely destroy all the enzyme. Cooling to room temperature immediately followed by allowing the flask to stand undisturbed in a vessel of cold water. During the cooling process the insoluble particles of powder settle to the bottom of the flask leaving a relatively clear yellowish solution of luciferin. The supernatant fluid is then decanted and is used as the stock luciferin solution. The oxidation of luciferin to oxyluciferin occurs spontaneously in the presence of oxygen in neutral and alkaline solutions, but in the absence of the enzyme such a spontaneous oxidation takes place without the production of light. This factor frequently plays a large part in the variations in the amount of light emitted from a given solution by reducing the available luciferin for the action of the luciferase. It has been customary, therefore, in the eleven series of experiments to use acid sea water in extracting the substrate, since acidity of the solution prevents the spontaneous oxidation. Later neutralization of the acid solution is effected in the actual experiments with N/10 NaOH, just previous to the addition of the enzyme.

In four series of experiments, another method of preventing spontaneous oxidation of luciferin was employed without the use of acid sea water. This method consisted of passing hydrogen through the luciferin solution for a considerable period of time immediately following the destruction of the enzyme. For this purpose a tall burette calibrated to 100 cc. was fitted with a rubber stopper at the open end. Through this stopper a U-shaped glass tube of small bore was fitted to allow the

escape of gas. By connecting the stop-cock of the burette to a hydrogen tank the contained oxygen could be replaced by hydrogen previous to the preparation of luciferin. The latter was then quickly poured into the burette filled with hydrogen, as soon after boiling as possible. The stopper was reinserted in the tube and hydrogen allowed to bubble through the solution until the whole mass had cooled to approximately room temperature. This procedure, however, lessens the amount of light emitted from the solution as will be seen from the data to be presented later.

In still other experimental series the hydrogen method was employed with sea water which had been neutralized to a pH 7.0. Very satisfactory results were obtained with this method of preparing the substrate.

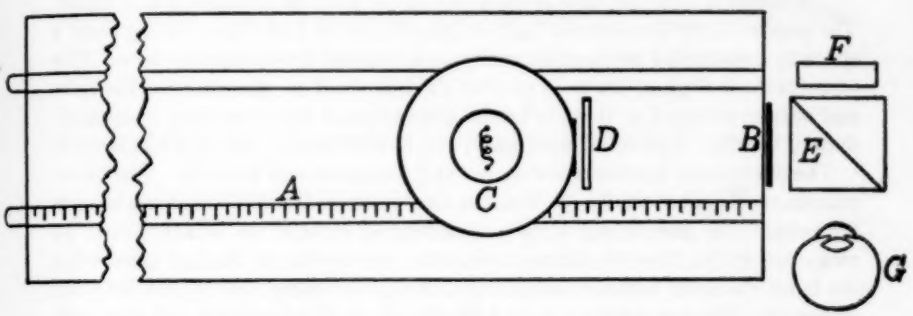


FIG. 2. Diagram of photometer bench. *A*, photometer bench; *B*, opal glass screen; *C*, chamber containing 21 C.P. lamp; *D*, No. 75 Wratten filter; *E*, comparison cube; *F*, mirrored vessel; *G*, position of observer.

The Photometric Method.

The measurement of luminescence of *Cypridina* solutions of varied concentrations is made possible by a comparison of the brightness of certain thicknesses of solution with the brightness emitted from a white surface illuminated by a light source of known intensity. The photometric method used in these experiments is an adaptation of that used by Harvey, 1925, and Morrison. The photometer bench (Fig. 2) consists of a track, one bar of which is marked off by divisions in mm. At one end of the bench (in this case at the 150 cm. mark) is placed a dull black screen with an opening in the center measuring 4.5 × 4.5 cm. square. Across this opening is fastened an opal glass screen. Care is taken that the screen shall be kept at the 150 cm. mark at all times. Behind this screen, on a movable standard, is a cylindrical dark chamber with an opening covered by a No. 75 Wratten blue green filter whose color value approximates most nearly that of the luminous solution of *Cypridina*. This chamber contains a small 6 volt 21 C.P. lamp of the type used in automobile headlights, so placed that illumination of the opal screen is complete. This chamber is attached to a sliding base so that the lamp can be

moved toward or away from the screen. Finally a plain black screen is fastened at the lower end on the calibrated scale of the photometer bench and the whole apparatus is entirely covered with a photographer's black cloth to prevent any reflections or radiations from extraneous sources from registering on the opal glass. In front of the opal glass screen is placed a comparison cube.[1] This cube is so constructed that alternately mirrored bands on the hypothenuse of one prism deflect a source of light issuing at right angles to the line of vision of an observer, while at the same time a beam of light in line with his vision is transmitted directly through the cube. Thus two different sources of light would appear to an experimenter as parallel adjacent bands whose intensities can easily be compared with only slight observational errors due to personal equations.

The source of reflected light is the 6 volt 21 C.P. lamp on the photometer bench. The source of the transmitted light is the solution of *Cypridina* coming from a specially constructed rectangular vessel placed behind the comparison cube. This vessel is made of glass, the front of clear glass, the back of opal glass and the sides and bottom mirrored so that the lateral dimensions of the vessel may be approximately infinite. The vessel used was 2 cm. in thickness by inside measurement.

The photometer lamp was always run at 2.3 amperes and 4.8 volts. The transmission coefficient of the No. 75 Wratten filter measured[2] 0.00573 with the lamp at 4.8 volts. The photometer lamp was calibrated without the Wratten filter by measuring with a Macbeth illuminometer the illumination of the opal glass, when the lamp was set at definite distances and the inverse square law applied for other distances. The test plate used with the Macbeth illuminometer had been calibrated by the Electric Testing Laboratories and gave a reflection factor of 0.79. By the formula, foot candles $\times$ constant, 1.076 (foot candles to millilamberts) $\times$ 0.79 (reflection factor) $\times$ 0.00573 (Wratten filter transmission factor) one obtains the brightness of the opal screen in millilamberts.

EXPERIMENTAL.

The experiments were always conducted in a dark room and with the aid of an assistant. The procedure of a typical experiment is here recorded in some detail as a matter of ease in understanding the variation which will be referred to later. It was customary to use 100 cc. N/10 HCl acid sea water and 1 gm. of powder for the luciferin extract. 10 cc. of this solution were carefully measured in a calibrated pipette and poured into the rectangular mirrored vessel.

[1] The comparison cube was kindly loaned by the Nela Research Laboratories of Cleveland.

[2] Kindly made by Dr. W. E. Forsythe of the Nela Research Laboratories for Dr. E. Newton Harvey.

To this was then added 5 cc. of sea water and 5 cc. of N/10 NaOH to neutralize the acid sea water. However, due to the buffers in the sea water and the alkaline nature of the *Cypridina* extract itself, the pH of such a solution ranged usually about 7.7. Such a solution contains about 0.1 gm. of *Cypridina* extract, although probably only a very small proportion of the powder used contains the luminous material, since the luminous organ comprises an extremly small percentage of the animal's body.

Immediately after preparing the vessel with the luciferin solution, 1 cc. of luciferase, containing 0.005 gm. of dry *Cypridina*, was quickly added to the luciferin solution and the time recorded. The vessel

TABLE I.

Series 3, Run 3.

Time	Distances (s)	s^2	$\frac{1}{s^2} \times 1000$	Logarithms
10	13.5	182.25	5.494	0.739
21	17.5	306.25	3.2679	0.514
31	22.0	484.00	2.0661	0.315
49	30.8	948.64	1.0548	0.023
65	45.8	2097.64	0.4784	$\bar{1}$.679
20	62.0	3844.00	0.2604	$\bar{1}$.415
36	87.8	7708.84	0.1298	$\bar{1}$.113
48	109.0	11881.00	0.0847	$\bar{2}$.927

Data calculated from observations in a typical experiment.

was rapidly agitated to facilitate mixing and placed immediately behind the comparison cube of the photometer apparatus. The observer then adjusted the photometer lamp until a match in intensities of the luminous bands on the comparison cube was obtained. At that moment the time in seconds and the distance in cm. on the bench scale were recorded by the assistant. The same procedure was repeated as the intensity diminished until the bands of light became too faint to read accurately. Very low intensities were discarded because of too great an error in comparison, and also frequently extremely high intensities were of little experimental value due to the fact that color differences between Wratten filter and the solution were accentuated.

Since intensity varies inversely as the square of the distance, the data obtained from such a series of records are tabulated by squaring the distance figures, and then obtaining the reciprocals of these squares and plotting the reciprocals against time. The reciprocals are taken as the arbitrary units used in the calculations. The graph in Fig. 1 is plotted from the data in Table I. These curves are comparable to Amberson's in showing that light dies out rapidly and follows the form of a pseudomonomolecular reaction. If the logarithms of the intensities are plotted against time, a straight line is obtained.

Immediately after a series of runs in any given set of experiments, the hydrogen ion concentration was determined by the colorimetric method for each solution used.

In any luminous solution of definite thickness prepared in as crude a manner as the *Cypridina* extract considerable absorption and some scattering of light must occur, due to particles held in suspension. Harvey, 1925, found this factor a very important one to consider in determining the lumens emitted by his emulsion of luminous bacteria. After recording the pH values, all the solutions of the same concentration were poured together and the light transmission determined directly as follows. The rectangular glass vessel with the mirrored sides and bottom and the opal glass back, which had been used for the experiments themselves, was filled with water and placed in front of the photometer lamp with the Wratten filter in place. The Macbeth illuminometer, also fitted with a No. 75 Wratten filter on the lamp side, was placed in front of the vessel of water and the inverse square scale on the illuminometer set at 10. The apparatus was then adjusted until a match in the fields of the illuminometer was obtained. This match with pure water represents 100 per cent transmission. The water in the vessel was then replaced by the *Cypridina* solution to be measured, and without changing the position of the apparatus the illuminometer lamp was moved back and forth until a match of the fields was obtained. The inverse square scale reads directly, and consequently gives the percentage transmission of the *Cypridina* solution. Readings can be duplicated to about 5 per cent for the low concentrations used. Since the vessel used for measuring the transmission of the solution is 2 cm. in thickness, and since the purpose of

the experiments is to determine the total light emitted from a 1 cm. cube of solution containing a definite amount of luminous powder, the thickness of the solution has to be taken into consideration and a correction made to obtain the transmission of a solution 1 cm. thick (T_1) by means of the formula

$$T_1 = \sqrt[n]{T_n}$$

in which T_n represents the thickness, n, of a solution used.

The determination of the amount of light emitted by 1 gm. of *Cypridina* powder is computed from the tabulated data as follows. It is necessary first to find the value in millilumens over 1 sq. cm. of the measuring vessel (2 cm. thick) for 1 arbitrary unit on the photometer scale, which will be constant for all experiments. If one obtains 2.1 foot candles of illumination of the opal glass for 2.5 arbitrary units, and since 1 millilambert in brightness equals 1 millilumen for every sq. cm., this value can be converted to millilumens per sq. cm. by the formula 2.1/2.5 foot candles $\times$ 0.00573, the transmission factor for the No. 75 Wratten filter, $\times$ 0.79, the reflection factor for the test plate, $\times$ 1.076, the conversion factor of foot candles to millilamberts. Solution of this formula gives 0.00408 millilumens per sq. cm. for 1 arbitrary unit. The total area of the decay curve (Fig. 1) is an index of the total amount of light emitted by the solution; this amount can be determined graphically by counting the squares and fractions of them. The straight line plotting of the logarithms is made to determine whether the experiment is progressing properly and also to determine the point of initial brightness of the solution at time 0. One square on the original plotting is equal to ½ an arbitrary unit for every 10 seconds, determined from actual readings (Fig. 1, lower left square), hence $k = 0.00408/2$ or 0.00204 millilumens per sq. cm. for 10 seconds. But this factor is the brightness for a solution measuring 2 cm. thick which shows some absorption. Correcting for 1 cm. and taking into account the transmission of the solution by the formula $T_1 = \sqrt[n]{T_n}$, where the transmission (T_2) in a typical experiment is 64 per cent, one obtains

$$T_1 = \sqrt{0.64}, \text{ or } \log T_1 = \frac{\log T_2}{2}$$

$T_1 = 0.80$ or 80 per cent transmission.

To determine the brightness of a solution 1 cm. thick with no absorption, ($\bar{B}_1$), the formula

$$B_n = \frac{\bar{B}_1 (T_n - 1)}{\log e \; T_1}$$

is used, in which B_n is the brightness of a solution n cm. thick, and T_n is the transmission of a solution n cm. in thickness. Applying this formula to the typical experiment

$$0.00204 = \frac{\bar{B}_1 (.64 - 1)}{\log e \; .80} \text{ or } 0.00204 = \frac{\bar{B}_1 (- .36)}{- .223} \text{ or } \bar{B}_1 = 0.001263 \text{ millilumens per sq. cm.}$$

This value can be easily converted from a surface brightness in cm. squares to a cube emitting light in all directions by multiplying by 4, which gives 5.052×10^{-6} lumens, the value from 1 cc. of solution of perfect transparency calculated for 10 seconds and corrected for 64 per cent transmission. Taking 37.5 area squares or arbitrary squares derived from experimental plottings in a typical run, and solving for total light emission per gm. of *Cypridina* in a solution containing 0.01 gm. of powder per cc. of solution, one obtains

$$0.005052 \times 10 \times 37.5 = \frac{1.895}{0.01} \times 10^{-3}$$

or 0.1895 lumens per gm. of powder per second.

Effect of Luciferase Concentration upon Amount of Light.

Table II contains data from three series of experiments totalling 32 runs with different concentrations of luciferase. It is understood, of course, that in an impure solution such as luciferase the actual concentration of enzyme is undetermined, but the relative concentrations are known. The velocity constants as determined from curves plotted for these series are tabulated in Table II. In one case the ratios are almost exactly the theoretical value. Amberson's conclusion that "the velocity of reaction is very nearly proportional to enzyme concentration" is supported by the present work.

Inspection of the data from the records of lumen-seconds per cc. of *Cypridina* solution (squares) shows considerable variation. These

variations in total light emission from solutions prepared in as nearly identical a manner as were the experimental solutions are not easily

TABLE II.

Amount of Light and Enzyme Concentration.

Enzyme concentration (C) in cc.	k	$\frac{k_2}{k_1}$	Squares	T_2	T_1	$\bar{B}_1 \times 4$	Lumens	Averages
				per cent	*per cent*		*gm. per sec.*	
Experiment III								
1	1.5		33.5	64	80	5.075×10^{-6}	.3396	
1	1.875		37.5	"	"	"	.3806	.356
1	1.86	1.58	34.25	"	"	"	.3476	
2	3.0		35.5	"	"	"	.3603	.3121
2	2.5		27.0	"	"	"	.2740	
Experiment IV								
1	1.80		46.6	74	86	4.75×10^{-6}	.4427	
1	1.55		40.6	"	"	"	.3857	.3844
1	1.83		34.2	"	"	"	.3249	
2	3.87	2.11	37.4	"	"	"	.3553	
2	2.70		38.6	"	"	"	.3667	.3591
2	4.40		37.4	"	"	"	.3553	
Experiment VI								
1	1.17		39.3	72	84.8	4.107×10^{-6}	.3776	
1	1.22		41.0	"	"	"	.3907	.3684
1	1.075		35.37	"	"	"	.3369	
2	1.69	1.64	33.4	"	"	"	.3182	
2	2.02		40.88	"	"	"	.3907	.3406
2	2.02		33.0	"	"	"	.3229	

The constant (k) of the reaction velocity is determined from the slope of the logarithmic plotting.

k_2/k_1 represents the ratio of the average velocity reaction slopes where k_2 is the velocity constant, when double amounts of enzyme are used.

Each cc. of enzyme solution contained 0.005 gm. of dry *Cypridina* powder and was added to 20 cc. of luciferin solution.

T_2 and T_1 are transmissions for 2 cm. and 1 cm. thick solutions; $\bar{B}_1 \times 4$ is explained in the text.

explained. Taking averages in all cases recorded, the total emission of light is about the same, but somewhat less, when the concentration

of luciferase is doubled. This is seen in the total light emission recorded as lumens per second per gm. of dry *Cypridina* which for 5 runs averages 0.3777 for concentrations designated as *C* and 0.3507 for 2*C* concentrations of luciferase. If the amount of light were independent of the luciferase concentration, these values should be the same.

Effect of Luciferin Concentration upon Amount of Light.

Seven series of experiments totalling 60 runs were made with variations in the concentration of luciferin from 2 gm. to 0.5 gm. of powder per 100 cc. water. Acid sea water, neutral sea water and distilled water saturated with hydrogen were used to prepare the luciferin. The data of three typical series are tabulated in Table III. Again considerable variation occurs in the lumen-seconds (squares) emitted per cc. of solution with the same concentration of luciferase. With higher concentrations of luciferin, the squares of light emitted are about twice as great. The total light emission recorded as lumens per second per gm. of dry *Cypridina* averages about the same in the different experiments with some tendency for the weaker luciferin concentrations to give relatively more light.

With a constant concentration of enzyme the velocity constant should be the same for a wide range of substrate concentrations, and the straight line plottings should be parallel, or nearly so. This expected relationship, however, did not appear in any of the graphs. Similar anomalous results occur also in Amberson's records. It is obvious, then, that the luciferin-luciferase system represents an exceptional case of reaction velocity.

While this work primarily is concerned with the amount of light emitted from 1 gm. of *Cypridina* powder, it is, nevertheless, important to interpret the curves which indicate the nature of the enzyme reaction under investigation. Why do velocity constants vary for the different concentrations of substrate? Without doubt the oxidation of luciferin is a monomolecular reaction in a heterogeneous system, since luciferase is a colloid and probably a protein (Harvey, 1920). It not infrequently happens that the change in a chemical reaction is less, the greater the initial concentration of the substrate (Hoeber), hence the reaction velocity cannot be defined by a definite constant to

signify the change in the two concentrations. Reactions of this sort are encountered in the inversion of cane sugar by invertase (Hudson),

TABLE III.

Amount of Light and Substrate Concentration.

Substrate concentration in gm. *Cypridina* per cc.	k	$\frac{k_2}{k_1}$	Squares	T_2	T_1	$\overline{B}_1 \times 4$	Lumens	Averages
				per cent	*per cent*		*gm. per sec.*	
Experiment XI								
.005	2.4		41.5	59	76.8	5.273×10^{-6}	.4376	
"	2.54		40.3	"	"	"	.4249	.390
"	2.43		34.29	"	"	"	.3616	
"	2.72		32.0	"	"	"	.3374	
.0025	2.8	1.16	19.83	89	94.3	4.369×10^{-6}	.3465	
"	2.98		18.00	"	"	"	.3145	.3156
"	3.2		16.70	"	"	"	.2920	
"	2.8		17.71	"	"	"	.3095	
Experiment XVIII								
.0025	1.47		7.62	75	86.6	4.713×10^{-6}	.1436	
"	1.25		7.007	"	"	"	.1332	
"	1.35		7.33	"	"	"	.1382	.1398
"	1.36		7.66	"	"	"	.1444	
.00125	1.6	1.26	4.09	92	95.9	4.286×10^{-6}	.1402	
"	1.6		5.02	"	"	"	.1721	
"	1.73		4.42	"	"	"	.1515	.1519
"	2.0		4.20	"	"	"	.144	
Experiment XVII								
.01	1.17		92.08	74	86	4.75×10^{-6}	.4373	
"	1.04		83.39	"	"	"	.3961	
"	1.00		91.17	"	"	"	.4330	.417
"	0.9		84.54	"	"	"	.4015	
.005	1.5	1.53	44.20	84	91.6	4.464×10^{-6}	.3946	
"	1.58		54.59	"	"	"	.4874	.439
"	1.57		47.81	"	"	"	.4268	
"	1.48		50.12	"	"	"	.4474	

Symbols the same as in Table II.

of grape sugar by zymase (Euler), and in the catalysis of H_2O_2 by platinum sol studied by Bredig and his students (Bredig and von Berneck). Hence the phenomenon is not peculiar to enzyme reactions

alone, and seems to be associated with the physical nature of the reactants.

Study of the data of this paper gives no evidence that the reaction is altered greatly by variation in pH between 6.6 and 8.4. In one series, at pH 6.8 an average of 0.508 lumens was obtained, while a series run at pH 8.2 emitted an average of 0.445 lumens. Consideration of all the data obtained does not justify the conclusion that the differences in velocity are due solely to pH differences. Salts can be excluded from consideration, since in a solution made up with sea water the additions of small amounts of *Cypridina* solution would not greatly affect the total salt content. The solutions were always brought to a room temperature of about 20°C. Measurements of viscosity between solutions containing 10 cc. and 5 cc. luciferin respectively gave a difference in magnitude of approximately 1 per cent. This variation is negligible. All these factors can, therefore, be excluded as the cause of the anomaly in velocity constants for different luciferin concentrations.

In the work with catalysis of metal sols, the so called microheterogeneous systems, catalytic activity is increased in weaker substrate solution, whereas in macroheterogeneous systems where the dispersion is less extensive the reaction follows the theortical proportionality. It is quite probable that in suspensions and colloids the substrate adsorbs to the surface of the enzyme in greater degree from the more dilute solutions. So many factors are involved in the enzyme reactions among colloidal solutions that an exact formulation of the kinetics has never been satisfactorily made, but as a working hypothesis it may be assumed that the greater velocity constant with weak luciferin concentration is associated with greater adsorption from this concentration.

SUMMARY.

1. A photometric method was devised for measuring the intensities of light emitted per cc. of luciferin solution and calculating the amount of light emitted per gm. of dried *Cypridina* powder. A total of 128 runs was made and the data are incorporated in this report.

2. The maximum amount of light emitted from 1 gm. of powder under the experimental conditions was 0.655 lumens. Different samples of powder vary greatly in amount of light production.

3. When the concentration of substrate is doubled, nearly twice as

much light is emitted, or an average ratio $2C/C$ of 1.86. Calculations of total light emissions *per gm. of powder* at different concentrations indicate that slightly more light is produced from the smaller concentrations. The maximum amount of light was produced by the solutions made with neutral sea water and averaged 0.445 lumens. The least light was obtained from solutions in distilled water saturated with hydrogen. The technique allows too rapid spontaneous oxidation prior to the saturation with hydrogen. The maximum amount of light from such experiments was only 0.077 lumens. Acid sea water solutions subsequently neutralized gave an average maximum of 0.386 lumens per gm. of powder per second.

4. When the concentration of enzyme is doubled, approximately the same amount of light is produced by both concentrations, although the stronger concentrations are slightly less effective than weaker ones. This undoubtedly is due to the colloidal nature of the enzyme and is a function of surface rather than of mass. In dilute solutions greater dispersion probably allows for greater adsorption to the surface of the enzyme. The average maximum amount of light produced in the series of enzyme experiments is of the magnitude 0.56 lumens per gm. of powder.

I wish to express my deep indebtedness to Dr. E. Newton Harvey under whose direction this study was undertaken, and to thank Mr. Stanton M. Hardy for the great help he rendered as my assistant.

I am also greatly indebted to Dr. Harvey for an abundant supply of *Cypridina hilgendorfii* Müller, the Japanese ostracod, the organism which has been used for the source of light in all the experiments.

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ON THE QUANTA OF LIGHT PRODUCED AND THE MOLECULES OF OXYGEN UTILIZED DURING CYPRIDINA LUMINESCENCE.

By E. NEWTON HARVEY.

(From the Physiology Laboratory, Princeton University, Princeton.)

(Accepted for publication, April 26, 1927.)

The primary effect in a photochemical reaction is the absorption of a quantum ($h\nu$)[1] of light energy with the conversion of a single molecule into reaction products. Later, secondary effects, such as the production of a catalyst by the light, may obscure the quantum relation, resulting in much greater chemical change than 1 quantum per molecule. On the contrary, not every quantum of light absorbed by a solution may result in chemical change, but there may be conversion into heat. In this case several quanta per molecule will be absorbed. As a matter of fact photochemical reactions are few in which the ratio of quanta absorbed to molecules decomposed is 1 (Taylor (1)).

In the reverse process, the emission of light through chemical change, chemiluminescence, we may also suppose as the primary process that 1 molecule undergoing change emits 1 quantum of light energy. Is light an invariable accompaniment of each molecular change? The few studies of chemiluminescence from this point of view show that change in many molecules occurs before 1 quantum is emitted. Thus, Haber and Zisch (2), studying the reaction of Na vapor and Cl at low pressures, observed the quanta of sodium *D* line emitted to be far less than the number of molecules of NaCl formed. This means that of the collisions between Na and Cl resulting in NaCl formation only a few emit light. It is obvious that this is an important point and should be studied further.

The present paper reports the results of an attempt to determine if a molecule of *Cypridina* luciferin, the oxidizable substance of the

[1] A quantum of light is the constant, h, equivalent to 6.554×10^{-27} erg. sec., times the frequency, ν, of the light in question.

luminous crustacean, *Cypridina hilgendorfii*, emits 1 light quantum on oxidation in presence of luciferase. As luciferin cannot be obtained pure and consequently weighed out with accuracy, it is necessary to measure the oxygen rather than the luciferin used up during luminescence and to assume an oxidative dehydrogenation of luciferin, for which there is considerable evidence, thus:

$$\text{luciferin} + 1/2\ O_2 = \text{oxyluciferin} + H_2O.$$

From this equation, which accounts for luminescence if luciferase is present, it is evident that 16 gm. or 3.03×10^{23} molecules of oxygen will oxidize a molecular weight of luciferin. If we measure the amount of oxygen consumed and the total amount of light produced by a given weight of *Cypridina*, the mg. of oxygen per lumen or the molecules of oxygen per quantum can be stated.

The total light produced is measured by direct photometric comparison of the brightness of a given depth of luminescent *Cypridina* solution with a surface whose brightness can be varied by known amounts. As the brightness of *Cypridina* luminescence decays with time, a curve of brightness against time is drawn, whose area will give the total amount of light produced in lumens, after allowing for absorption of light in the yellowish solutions. Details of the measurement are given by Stevens,[2] whose work shows that, with the same amount of luciferin, the total light is somewhat greater with smaller concentrations of luciferase. The total light is about proportional to the mass of luciferin but varies with other factors so that no great accuracy is claimed, even for samples of the same dried *Cypridina* material. In fact, only the order of magnitude of the oxygen per lumen can be given, since measurement of oxygen consumption presents the greatest difficulties.

The difficulty in measuring oxygen consumption by differential manometer methods is due to the fact that luciferin solution is prepared by making a boiling water extract (which destroys luciferase) of dried *Cypridina* and cooling the extract quickly. Such a solution is not in equilibrium with any *known* pressure of oxygen and cannot be brought into equilibrium with air without the oxidation of a consider-

[2] See preceding paper of this journal.

able and unknown amount of luciferin without luminescence. It is therefore necessary to keep luciferin solution away from oxygen until ready to measure its oxygen consumption. The method finally adopted is to allow the luciferin luminescence to indicate the complete oxidation of the luciferin, as explained in connection with Fig. 1.

In Vessel *B*, of 25 cc. capacity, 4 cc. luciferase is placed (1 per cent dried *Cypridina* solution) and 0.5 cc. mercury to serve as a stirrer. With Cocks *F*, *C* and *D* open, and *E* closed, a stream of hydrogen freed of oxygen by passage over red hot platinized asbestos in a quartz tube is passed through the vessel for 1 hour, thus removing all oxygen. The hydrogen passes through lead tubing (since rubber tubing

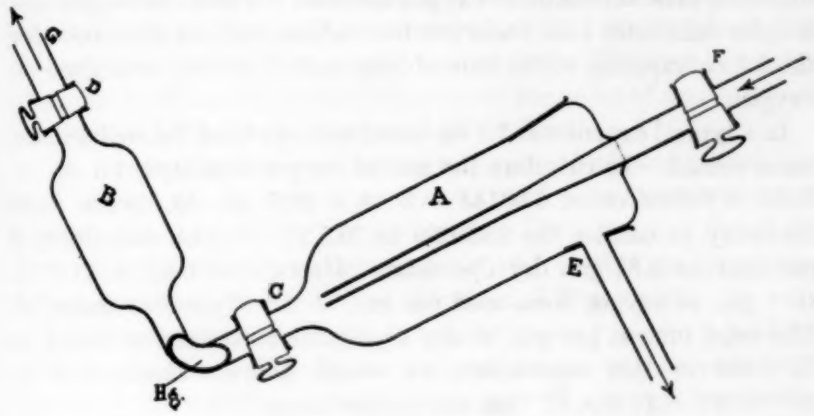

FIG. 1.

is permeable to oxygen) sealed to the *F* end of the apparatus with de Kohtinsky cement. Cocks *C* and *D* are then closed.

A solution of luciferin is then made by extracting 2 gm. of dry *Cypridina* with 50 cc. hot water (4 per cent solution) and immediately decanting into Vessel *A*. With Cocks *F* and *E* open, a stream of pure hydrogen is now passed through *A* for 1 hour, when *E* is closed. By opening *C* and *D* carefully, luciferin is now mixed with luciferase and *B* completely filled with fluid, when all cocks are closed and the vessel disconnected from the supply of hydrogen. There will be no luminescence in Vessel *B* since oxygen is absent.

The remaining luciferin in Vessel *A* can now be taken to the pho-

tometer bench, mixed with luciferase in presence of oxygen and the total light produced measured as described.[3] In a typical satisfactory experiment it amounted to 0.101 lumens per gm. of dried *Cypridina* material.

Vessel *A* is then filled with distilled water saturated with air at 22°C. Each cc. of distilled water will dissolve 0.006 cc. oxygen (measured at N. T. P.) By opening Cocks *C* and *D*, small amounts (0.1 to 0.2 cc.) of water are allowed to flow into *B*, displacing a corresponding amount of luminescent solution into *G* which can then be measured in *G*. After each admission the fluid in *B* is thoroughly mixed by shaking the mercury back and forth and luminescence noted. Luminescence following each admission of oxygen becomes less and less bright and is quite faint after 1 cc. water has been added, making allowance for the 0.1 cc. capacity of the bore of Stop-cock *C*, which contained no oxygen.

In a typical experiment 1.1 cc. water was admitted before luminescence ceased. To calculate the gm. of oxygen admitted: 1.1 cc. $\times$ 0.006 = 0.0066 cc. $\times$ 0.00143 = 9.44×10^{-6} gm. of oxygen were necessary to oxidize the luciferin in 20.5 cc. solution containing 4 per cent or 0.82 gm. dry *Cypridina*. Hence $9.44/0.82 = 11.5 \times 10^{-6}$ gm. of oxygen were used per gm. of dry *Cypridina* material. The total lumens per gm. of dry *Cypridina* material was found to be 0.101 in this experiment, as stated above. Hence $11.5 \times 10^{-6}/0.101 = 11.4 \times 10^{-5}$ gm. oxygen per lumen.

It is quite obvious that this method of determining oxygen cannot give very accurate results since the end-point is not very sharp. It is also certain that when the admission of oxygen results in only faint luminescence there must be an excess of oxygen present. This is because luminescence intensity is really dependent on reaction velocity and not on concentration of luciferin (Amberson (3)). With considerable luciferin and little oxygen, luminescence will be faint because the low oxygen concentration is slowing reaction velocity; with little luciferin and considerable oxygen, luminescence will be faint because the low luciferin concentration is slowing the reaction velocity. The latter statement corresponds to the condition when we are nearing the end-point in admitting oxygen. In fact it is possible to show that

[3] I am deeply indebted to Mr. K. P. Stevens for making these measurements.

after 1 cc. of water has been admitted to Vessel *B*, there is excess oxygen present, since fresh oxygen-free luciferin solution introduced into *B* will give a fair luminescence.

The results tell us only that less than 11.4×10^{-5} gm. oxygen per lumen are necessary for a lumen of luminescence. It will be noted also that the assumption is made that all the oxygen admitted to the luciferin solution is used in oxidizing luciferin, and that there are no other easily oxidizable compounds present. I have endeavored to test this point by adding to the dried *Cypridina* powder an equal weight of dried powdered pill-bugs (*Oniscus*) which might supply easily oxidizable substances but not luciferin, and then determining oxygen consumption. The runs with added dried *Oniscus* showed about 25 per cent more oxygen consumed, so that this factor can play no very great part in the oxygen consumption. However, the most that can be claimed is a determination of the order of magnitude of oxygen consumption per lumen of light emitted.

The average of eleven experiments completed without mishap was 10.6×10^{-5} gm. of oxygen per lumen, with extreme variations of 5×10^{-5} and 15.5×10^{-5} gm. per lumen. The average of five of the later more satisfactory experiments was 11.7×10^{-5} gm. per lumen with a maximum variation from the mean of 25 per cent.

Upon the basis of 11.4×10^{-5} gm. of oxygen per lumen of luminescence, we may calculate the molecules of oxygen per quantum with the aid of the visibility of radiation curve.[4] The energy distribution in the *Cypridina* luminescence spectrum has recently been determined by Coblentz and Hughes (4). The maximum emission is at $\lambda = 0.48\mu$ and the visibility curve tells us that 1 lumen of $\lambda = 0.48\mu$ light is equivalent to 0.01 watt or 0.01×10^{7} ergs per second. A quantum ($h\nu$) of $\lambda = 0.48\mu$ light is equal to 4.1×10^{-12} ergs so that 1 lumen of $\lambda = 0.48\mu$ light contains 2.45×10^{16} quanta. As 11.4×10^{-5} gm. of oxygen contain 2.16×10^{18} molecules and will oxidize 4.32×10^{18} molecules of luciferin we see that 88 molecules of oxygen or 176 molecules of luciferin must undergo change in order to produce 1 quantum of light of $\lambda = 0.48\mu$.

For reasons stated above the actual value is probably somewhat less than this. A conservative statement might be that about 50 mole-

[4] See data on light units, Trans. Illuminating Engineering Soc., 1922.

cules of oxygen or 100 molecules of luciferin react to produce 1 quantum of *Cypridina* luminescence. *Certainly more than 1 molecule of oxygen per quantum is necessary.*

Accepting 50 molecules of oxygen per quantum or 6.48×10^{-5} gm. per lumen of luminescence, a few other orders of magnitude can be calculated, based on the luminescence equations (Harvey (5)):

$$\text{luciferin } (LH_2) = \text{oxyluciferin } (L) + H_2 - 15 \text{ Cal.}$$

$$H_2 + 1/2\ O_2 = H_2O \text{ (liquid)} + 69 \text{ Cal.}$$

The heat of oxidation of luciferin is therefore 54 Calories per gm. mol, justified on the general rule for heats of combustion of organic compounds (Thornton (6)). The gm. molecular heat equivalent of the quantum of $\lambda = 0.48\mu$ light is 59.3 Calories, obtained from the relation U (in Calories) = N (Avogadro constant) $h\nu$, but we are as yet uncertain whether this relationship can be applied to chemiluminescent reactions.

If 16 gm. of oxygen oxidize luciferin with production of 54,000 calories of heat, 6.48×10^{-5} gm. of oxygen will evolve 2.19×10^{-1} calories and correspond to the emission of 1 lumen of $\lambda = 0.48\mu$ light, equivalent to 2.39×10^{-3} calories. Hence the efficiency, *energy in light/heat of reaction*, is about 1.1 per cent. The rise in temperature in a 4 per cent solution of dried *Cypridina* should be in the neighborhood of 0.001°C.

The oxygen necessary to combine with the luciferin in 1 cc. of a 4 per cent solution of dried *Cypridina*, on the basis of 50 molecules per quantum, is approximately 0.3×10^{-6} gm. or a 0.00001 molecular solution. A 4 per cent *Cypridina* solution must therefore contain luciferin in approximately 0.00002 M concentration.

The results show beyond any doubt that more than 1 molecule of luciferin must react to produce 1 quantum. Luminescence is not therefore an invariable accompaniment of luciferin oxidation. This is to be expected if luciferase is the source of the light, which results from transfer of the energy of oxidation of luciferin to some of the luciferase molecules, exciting them to luminescence (Kautsky and Zocher (7)). Spontaneous oxidation of luciferin is proceeding and only those molecules of oxidizing luciferin can transfer their energy which occupy special positions as regards the luciferase molecules.

SUMMARY.

A study of the oxygen consumed per lumen of luminescence during oxidation of *Cypridina* luciferin in presence of luciferase, gives 11.4 $\times 10^{-5}$ gm. oxygen per lumen or 88 molecules per quantum of $\lambda = 0.48\mu$, the maximum in the *Cypridina* luminescence spectrum. For reasons given in the text, the actual value is probably somewhat less than this, perhaps of the order of 6.48 $\times 10^{-5}$ gm. per lumen or 50 molecules of oxygen and 100 molecules of luciferin per quantum. It is quite certain that more than 1 molecule per quantum must react.

On the basis of a reaction of the type:

$$\text{luciferin} + 1/2\ O_2 = \text{oxyluciferin} + H_2O + 54\ \text{Cal.},$$

it is calculated that the total efficiency of the luminescent process, *energy in luminescence/heat of reaction*, is about 1 per cent; and that a luciferin solution containing 4 per cent of dried *Cypridina* material should rise in temperature about 0.001°C. during luminescence, and contain luciferin in approximately 0.00002 molecular concentration.

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THE KINETICS OF OSMOSIS.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Accepted for publication, April 27, 1927.)

The equilibrium conditions in osmotic pressure experiments have been thoroughly investigated both theoretically and experimentally. The kinetics of the process, however, has received little attention although the general theory is known. The process of osmosis is essentially one of diffusion of the solvent into the solution and so should follow in general the diffusion laws. In ordinary diffusion experiments, however, such as the solution of a solid or the diffusion of salt through a vessel of water, the solute is the component which moves while in osmosis it is the solvent. In the former case, since the number of solute molecules is ordinarily only a small fraction of the total number, the total number of molecules remains practically constant, while in osmosis, the total number of molecules in the solution changes during the experiment. It might be expected therefore that the equation for osmosis would differ slightly from that of diffusion of the solute since the terms containing the total number of molecules, *i.e.* the volume, which are constant in the ordinary diffusion formula are now variables. As will be seen this is the case experimentally.

The desired relation may be derived in a number of ways, but the following derivation, although not mathematically rigorous, appears to the writer to be the simplest.

Assume the solution separated from the solvent by a membrane permeable only for the solvent, as shown in Fig. 1. The mole fraction of the solute is assumed small and the solution is assumed to obey the laws of ideal solutions. Solvent will pass through the membrane from the pure solvent into the solution. The volume and hence the hydrostatic pressure on the solution will be increased and the process

will stop when the hydrostatic pressure equals the osmotic pressure. It is desired to know the quantity of solvent which passes through the membrane at any time.

According to the general law of diffusion (or flow) the rate of flow per unit area is proportional to the pressure gradient, or, in this case, to the pressure divided by the resistance; or

$$\frac{dv}{dt} \sim \frac{P}{R_1},$$

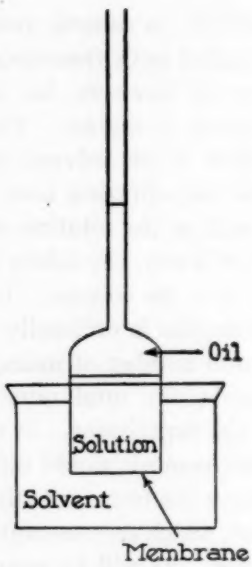

FIG. 1. Apparatus for the determination of the rate of osmosis.

in which v is the volume, t the time, P the pressure, and R the resistance offered to the flow.

In order to integrate this equation the variable terms must be expressed as functions of v or t. The pressure is evidently equal to the osmotic pressure, which tends to force the water in, less the hydrostatic pressure, which tends to force it out,

$$P = OP - HP.$$

The osmotic pressure is defined by the equation,[1]

$$OP = \frac{RT}{V_m}(-\ln(1-x))$$

which for dilute solutions reduces to

$$OP = \frac{RTx}{V_m} \tag{1}$$

where R is the gas constant, T the absolute temperature, x the mole fraction of the solute, and V_m the molal volume of the solvent in the solution.[2] Assume there are g gm. of solute of molecular weight M dissolved in V cc. of water. The mole fraction of the solute, then, will be the moles of solute divided by the total moles, or

$$x = \frac{\frac{g}{M}}{\frac{V}{V_m} + \frac{g}{M}}.$$

Since $\frac{g}{M}$ is assumed small in comparison to $\frac{V}{V_m}$, the equation may be written $x = \frac{gV_m}{MV}$. Substituting this value of x in (1),

$$OP = \frac{RT}{V_m} \cdot \frac{gV_m}{MV};$$

[1] *Cf.*, for instance, Washburn, E. W., Physical chemistry, New York, 1st edition, 1915, 155.

[2] Since we are interested only in the *amount* of water that diffuses into the system, *i.e.*, the amount that passes through the plane at the outside surface of the membrane, it is not necessary to consider the pressure gradient within the solution. The pressure may therefore be assumed proportional to the average pressure, *i.e.*, to the pressure that would exist if the solution were homogeneous. That this is so may be seen from the fact that the pressure gradient would depend on the diffusion coefficient, which does not affect the form of the equation but only the value of the constant. Mathematical proof of this statement may be found in the fact that if the equation is solved according to the general form of Fouriers theorem, which takes into account the pressure gradient and gives the amount of water which passes through a plane at any distance y, from the surface of the membrane, and y is then made equal to zero, the equation reduces to the same form as the integral of (1). *Cf.* Mellor, J. W., Higher mathematics for students of chemistry and physics, New York, 4th edition, 1913, 488.

or, since in any one experiment R, T, M and also g are all constant and may be combined into one constant P_o,

$$OP = \frac{P_o}{V}. \tag{2}$$

The hydrostatic pressure will be equal to the initial pressure, n', plus the additional pressure caused by the rise of the solution in the capillary. If 1 cc. increase in volume causes the liquid to rise K' mm., and f is the relative specific gravity of the liquid compared to mercury, then the hydrostatic pressure at any time expressed in mm. of mercury will be equal to $fn' + fK'(v - v_o)$, or $HP = n + K(v - v_o)$, where v is the volume at any time and v_o is the initial volume; n is the initial pressure expressed as mm. mercury; and K is the increase in pressure per cc. increase in volume, expressed also as mm. mercury.

Evaluation of R_1.— R_1 may be expressed in different ways depending on the mechanism assumed for the passage of water through the membrane. If the water is assumed to dissolve in the membrane and so pass by diffusion, R_1 is a function of the diffusion coefficient. If the water is assumed to flow through capillaries, then R_1 is a function of the size and number of the capillaries.

1. The Water Dissolves in the Membrane.

The thickness of the layer of solution for a cylindrical vessel with the membrane at one end will be $\frac{v}{\pi r^2}$, where r is the radius of the cylinder. If the thickness of the membrane is h then the total average distance the water has to diffuse will be $h + \frac{v}{2\pi r^2}$, and the total resistance offered to its flow per unit of area will be the distance times the specific resistance; or if R_m is the resistance offered by unit thickness of collodion and R_w the resistance offered by unit thickness of the solution, the total resistance $R_1 = hR_m + \frac{v}{2\pi r^2} R_w$. If the membrane is of such a nature therefore that the solvent can diffuse through it as rapidly or nearly so as through the solution, it is evident that the resistance offered by the membrane may be neglected, since the

distance passed through in the membrane is very small compared to the total distance, and $R = \frac{v}{2\pi r^2} R_w$. In the case of collodion membranes and most other artificial membranes the resistance offered by the membrane is enormously greater than that offered by the solution, so that the term representing the resistance of the solution may be neglected and $R = hR_m$. Since the diffusion coefficient is the reciprocal of the specific resistance, $R = \frac{h}{C}$ where C is the diffusion coefficient of the solvent in the membrane.

2. The Water Flows through Capillaries in the Membrane.

In the evaluation of the resistance given above the solvent was assumed to diffuse through the membrane in the same way as through the solution and the increase in resistance was ascribed to the difference in the rate of diffusion of the solvent molecules in the membrane and in the solution. There is some reason to believe, however, that collodion membranes at any rate may be considered as consisting of pores in a solid and that the water passes only through the pores. From this point of view the resistance offered by the membrane will be determined by Poiseuille's law.[3] The resistance offered by the solution can again be neglected. If there are p pores of radius r_1 per unit of surface, and they are assumed to be the same length as the thickness of the membrane, the quantity of water that will pass under unit pressure according to Poiseuille's law will be proportional to $\frac{pr_1^4}{h\eta}$, η being the viscosity, and the resistance offered to the passage of the water will be the reciprocal of this or $\frac{h\eta}{pr_1^4}$. Since for any one solvent and membrane η, p and r_1 are constant the resistance will be $\frac{h}{C}$ as before.

In either case, then, the total amount of water passing through will be proportional to $\frac{SC}{h}$ where S is the total surface of the membrane.

[3] *Cf.* Hitchcock, D. I., *J. Gen. Physiol.*, 1925–27, viii, 71.

Substituting these values of R and P, equation (1) becomes

$$\frac{dv}{dt} = \frac{CS}{h}\left[\frac{P_o}{v} - [n + K(v - v_o)]\right]. \tag{3}$$

C is the quantity of solvent that will pass through a unit area of membrane of unit thickness in unit time under unit pressure. Changes in the value of C are due then either to changes in the rate of diffusion in the membrane or to changes in the pore size or number, or the viscosity of the solvent, depending on which mechanism is assumed for the passage of the solvent through the membrane.

If there is no hydrostatic pressure on the solution at the beginning of the experiment the equation in this form predicts that the quantity of solvent passing through the membrane in the first few minutes will be proportional to the osmotic pressure of the solution. This relation has been shown to be true by a number of workers and has been used to measure the pressure in cases where the equilibrium value could not be obtained.[4]

At equilibrium no solvent passes through the membrane, *i.e.* $\frac{dv}{dt} = 0$, so that

$$\frac{P_o}{v_e} = n + K(v_e - v_o), \tag{4}$$

or

$$Kv_o = n + Kv_e - \frac{P_o}{v_e},$$

where v_e is the volume of solution at equilibrium.

Equation (3) may be integrated in a number of forms depending on which constants are used. Mathematically the simplest expression is obtained in terms of P_o, v_e and K. In order to obtain the equation in these terms the value of Kv_o, from equation (4), is substituted in equation (3). Collecting terms and simplifying, the equation becomes $\frac{dv}{dt} = \frac{CSP_o}{hv_e}\frac{(1 + bv)(v_e - v)}{v}$, where $b = \frac{Kv_e}{P_o}$, which, on integration, gives

$$C = \frac{2.3\, hv_e}{(1 + bv_e)SP_o t}\left(v_e \log \frac{v_e - v_o}{v_e - v} - \frac{1}{b}\log \frac{1 + bv}{1 + bv_o}\right); \tag{5}$$

[4] *Cf.* Findlay, A., Osmotic pressure, London and New York, 2nd edition, 1919.

or if $K = 0$, that is when the experiment is so arranged that the hydrostatic pressure is constant,

$$C = \frac{hv_e}{SP_o t}\left(v_o - v + 2.3\, v_e \log \frac{v_e - v_o}{v_e - v}\right). \quad (6)$$

TABLE I.

Rate of Osmosis 30°C.

Experiment 1.

$v_o = 3.0$ $P_o = 52.5$ $K = 2.28$ $n = 1.1$ $v_e = 6.2$ $b = .27$ $S = 10$ sq. cm.

T	v	$K_m \times 10^4$	$\frac{C}{k} \times 10^4$
hrs.	*cc.*		
0	3.0		
24	3.70	47.5	2.27
48	4.20	44.0	2.19
96	4.85	38.0	1.96
192	5.60	36.0	1.98

Experiment 2

3.2 cc. a "soluble" gelatin in thistle tube closed with collodion membrane.
$v_o = 3.2$ $P_o = 288$ $K = 0$ $n = 50$ $v_e = 5.85$ $S = 4.5$ sq. cm.

T	v	$K_m \times 10^4$	$\frac{C}{k} \times 10^4$
hrs.	*cc.*		
0	3.2		
24	3.75	42.5	1.53
48	4.15	40.0	1.53
96	4.72	38.7	1.62
192	5.22	32.0	1.51
300	5.85		

This condition would also be true if the solvent outside the membrane were replaced by a large volume of solution having a lower osmotic pressure than that of the solution inside. The osmotic pressure of the outside solution would enter into the equation in the same way as does the initial hydrostatic pressure, n, in equation (3).

A number of experiments were performed to test the accuracy of these equations, and they were found to hold within the experimental error.

Table I and Fig. 2 give the results of two such experiments. In the first experiment solutions of egg albumin in $M(NH_4)_2SO_4$ were placed in a rocking osmometer,[5] with the same concentration of ammonium sulfate outside, and left until equilibrium was established.

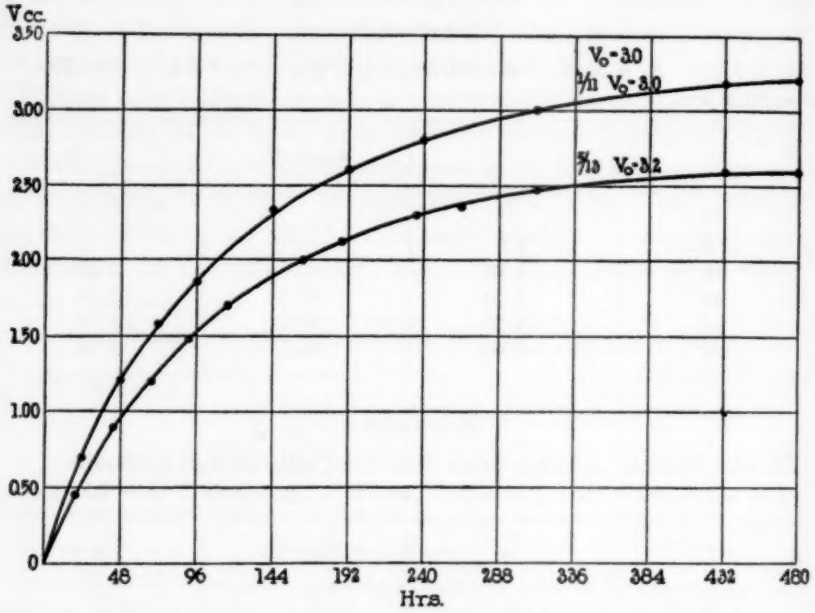

FIG. 2. The rate of osmosis of protein solutions in collodion membranes.

3 cc. of the solution was then placed in a tube closed with a membrane, the upper part of the tube filled with oil and the membrane immersed in the solution of ammonium sulfate with which the albumin solution had previously been in equilibrium. The pressure in the manometer was set at the equilibrium value and the system left for 2 days so that the permeability of the membrane might become constant. The manometer level was then lowered and the rise of the

[5] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1925–26, ix, 351; also 1926–27, x, 161.

oil in the manometer noted. The experiment was carried on in a water bath at 30°C. In the presence of this concentration of ammonium sulfate the osmotic pressure of albumin is nearly proportional to its concentration, so that it may be assumed to obey the ideal solution law. The ammonium sulfate also prevents bacterial growth. The second experiment was done in the same way except that a solution of "soluble" gelatin[5] was used. In this case the manometer tube was bent so as to be horizontal. There was therefore no change in pressure during the experiment and equation (6) should fit. The table shows in both cases that the monomolecular constant K_m given for comparison shows a regular decrease while the constants calculated by equations (5) and (6) do not vary outside of the experimental error. This was found to be the case in all of the experiments made. The monomolecular constant dropped slowly in every case.

In the first experiment the value of $\frac{C}{h}$ was found to be 2×10^{-4}. If the derivation given is correct this should be the cc. of water that will flow through 1 sq. cm. of the membrane in 1 hour under 1 mm. mercury pressure. At the end of the experiment the membrane was washed, filled with water and the rate of flow of water through it determined under 10 cm. mercury pressure. A value for the rate of flow of 1.5×10^{-4} cc. per hour per mm. mercury pressure was obtained, which agrees as well as could be expected with the figure calculated from the osmotic pressure experiment.

In this experiment the surface of the membrane is constant. In experiments with cells such as those of McCutcheon and Lucke[6] the surface increases during the experiment. If the water is assumed to diffuse through the membrane, the thickness of the membrane being constant, then $S \propto v^{\frac{2}{3}}$; or if the volume of the membrane remains constant $\frac{S}{h} \propto v^{\frac{4}{3}}$. If the water is supposed to flow through pores in the membrane and the increase in surface is due to enlarging the size of the pores, the thickness remaining constant, then $r^2 \propto S \propto v^{\frac{2}{3}}$ and $r^4 \propto v^{\frac{4}{3}}$. According to the first assumption the velocity should be very slightly slower than that predicted by the monomolecular formula and according to the second or third assumption it should be very

[6] McCutcheon, M., and Lucke, B., *J. Gen. Physiol.*, 1925–26, ix, 697.

slightly faster. In either case the deviation from the monomolecular formula would be noticed only in very accurate experiments.

SUMMARY.

It is shown that by combining the osmotic pressure and rate of diffusion laws an equation can be derived for the kinetics of osmosis.

The equation has been found to agree with experiments on the rate of osmosis for egg albumin and gelatin solutions with collodion membranes.

THE SWELLING OF ISOELECTRIC GELATIN IN WATER.

I. Equilibrium Conditions.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Accepted for publication, April 27, 1927.)

If a block of isoelectric gelatin is placed in water it will imbibe water and increase in volume. The writer and Kunitz (1) were able to measure the pressure with which this water was drawn into the block and found that it increases with the concentration of the gelatin. According to the phase rule the concentration (osmotic pressure) of a solution in the presence of the solid is independent of the amount of solid. It was suggested therefore that gelatin consisted of two or more fractions some of which were insoluble at low temperature while others were soluble and so could exert osmotic pressure. The swelling of isoelectric gelatin then becomes a process of osmosis just as Wilson (2) showed was the case for the swelling of gelatin in acid, except that in the case of isoelectric gelatin the osmotic pressure is not due to the ions of an electrolyte but to the presence of a soluble constituent of the gelatin itself. Water therefore enters the gelatin until the elastic pressure is equal to the osmotic pressure. The present paper is an attempt to apply this mechanism quantitatively to the swelling of gelatin.

The general behavior of gelatin when placed in water has been described by a number of investigators and in detail by the very complete experiments of Arisz (3). The more striking peculiarities may be briefly described as follows. In general the swelling increases with the temperature and with the concentration of gelatin. A block of gelatin concentrated by allowing the water to evaporate swells much more than a similar block made by allowing a solution of the same concentration to solidify. Thin films of gelatin reach a value which increases only slowly while large blocks do not give any indication of a maximum value but continue to swell until dissolved. At higher

temperatures there is less indication of an equilibrium value. If a block of gelatin is allowed to remain in water until it has stopped swelling and then is raised to a higher temperature in air for a short time under such conditions that there is no change in volume, it will swell rapidly when replaced in water at the first temperature. These peculiarities are similar to those of any substance when under an elastic strain, and can be readily accounted for at least qualitatively by the mechanism of swelling stated above. When the block is placed in water, water enters owing to the osmotic pressure of the solution in the block. The fibres of solid material are thereby forced apart and the force with which they attempt to return to their original position opposes the entrance of the water. At the same time the osmotic pressure is decreasing owing to dilution. When the elastic force equals this osmotic pressure the process stops. If the fibres have been stretched beyond their elastic limit, however, or if the force is applied for too long a period of time the fibres become fatigued and the elastic force is lessened so that more water enters. As is the case with any elastic body therefore a true equilibrium value is never reached. The osmotic pressure increases with the temperature and at the same time the amount of solid material decreases and also probably becomes less elastic so that swelling increases very rapidly as the temperature rises. In a thin film the whole mass becomes filled with water before the fibres become fatigued whereas in a large block the outside layers, which swell first, become fatigued and take up more water before the water has diffused into the inner layers. This "secondary swelling" therefore overlaps the primary so that in the case of large blocks there is no indication of a maximum value. This mechanism will be discussed more in detail under the kinetics of the swelling process. When a block which has swollen at a low temperature is raised to a higher temperature the elasticity is destroyed and it therefore swells again when replaced in water. When the gelatin is concentrated by evaporation it decreases in size and is therefore under an elastic strain just as when it swells except that in the case of loss of water the elastic force is in the same direction as the osmotic pressure instead of opposite to it. The block therefore swells more than a similar one which is not under elastic strain at this concentration.

Experimental Procedure.

In order to avoid the difficulty of working with very thin pieces of gelatin the gelatin was coated on glass rods about 0.2 by 15 cm. The rods were weighed, dipped in gelatin of the desired strength at a temperature of 40°C., weighed again in order to determine the amount of gelatin in the film and then placed in stoppered tubes containing a strip of wet filter paper in order to prevent loss of water by evaporation.[1] The tubes were then put in the cold room at 5°C. Arisz found that gelatin swells more if placed in water at a low temperature immediately after solidifying than if kept at the same temperature in air for a time before placing in water. After the 1st day no further change occurred. These experiments were repeated and confirmed except that a change was noted for the first 3 days. All the gelatin used in these experiments was therefore kept at 5° for 3 days before it was placed in water. If, as the writer assumes, solid gelatin contains a saturated solution of the material forming the network this is exactly the behavior expected since time is required to reach the equilibrium value. The experiment is analogous to that of Loeb (5) who found that the osmotic pressure of a gelatin solution cooled from 70° to 37° was higher at first than that of a solution raised from 10° to 37°, but later became the same. At the beginning of an experiment the rods were weighed again and then placed in water at 5° and weighed at intervals after wiping dry with soft filter paper. Since warming even for a short time destroys the elasticity, it is necessary to weigh at the same temperature as that at which the swelling occurred. Some

[1] Schroeder found that gelatin which was in apparent equilibrium with water lost weight when suspended in saturated water vapor at the same temperature and this has been considered as contrary to the second law of thermodynamics. As Bogue (4) has pointed out, however, the force of gravity is acting on the gelatin in the air but not in the water and if the gelatin is supposed to consist of a network of capillaries, this is sufficient to account for the observation. Suppose a fine capillary is dipped into water and after the water has risen in the tube, the tube is removed and suspended vertically just above the surface of the liquid. A drop will be formed at the bottom of the tube and since this drop has a convex surface its vapor pressure will be higher than that of the body of water. Water will evaporate from the drop until the surface of the water at the bottom of the capillary is no longer convex.

experiments were also made with slides dipped in gelatin and with thin slices of gelatin cut from a cylinder. Spheres of gelatin made by dropping liquid gelatin into cold toluene were also used.

For convenience in the calculation and presentation of the results they have been expressed in terms of gm. of water per gm. of gelatin.

Measurement of the Swelling Pressure.—The pressure with which the water tends to diffuse into the gelatin was measured by the writer and Kunitz (1). The measurements were extended to higher concentrations using the same method as described. The results of these

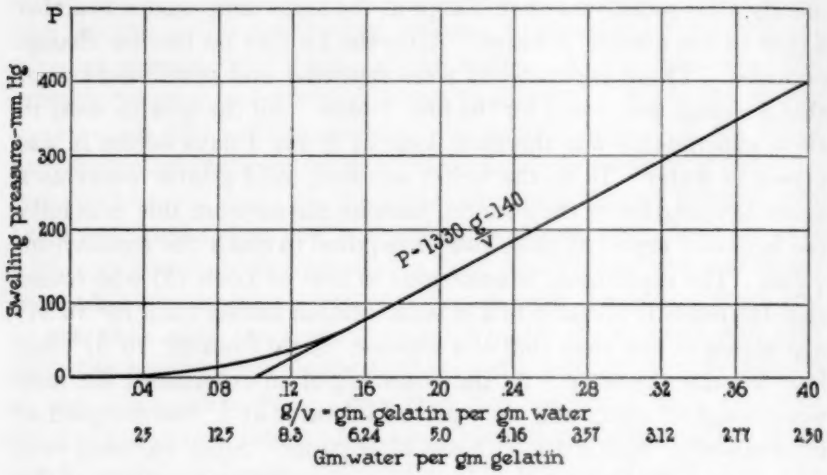

FIG. 1. Concentration-swelling pressure of gelatin at 10°.

measurements of the swelling pressure of isoelectric gelatin at 5° are shown in Fig. 1.

These pressures were obtained with gelatin that had been made up to the concentration noted while liquid and it seemed quite possible that a different pressure would be obtained when the concentration was changed by a swelling of the solid gelatin. In order to test this point a Chamberland filter was coated with collodion and then with gelatin containing 40 gm. per 100 gm. of water. The thimble was then placed in water for 12 hours at the end of which time the concentration was 30 gm. of gelatin per 100 gm. of water. The filter and

gelatin were then removed from the water, the inside filled with water and a manometer tube attached as previously described (1). At first no pressure was obtained as the swelling pressure was balanced by the elasticity of the gelatin. As the elasticity decreased with time, however, the pressure gradually rose and after 4 days remained constant at 255 mm.Hg pressure. This is the same, within the experimental error, as the value obtained with 30 per cent gelatin which had been prepared by adding water to the melted gelatin. The effect on

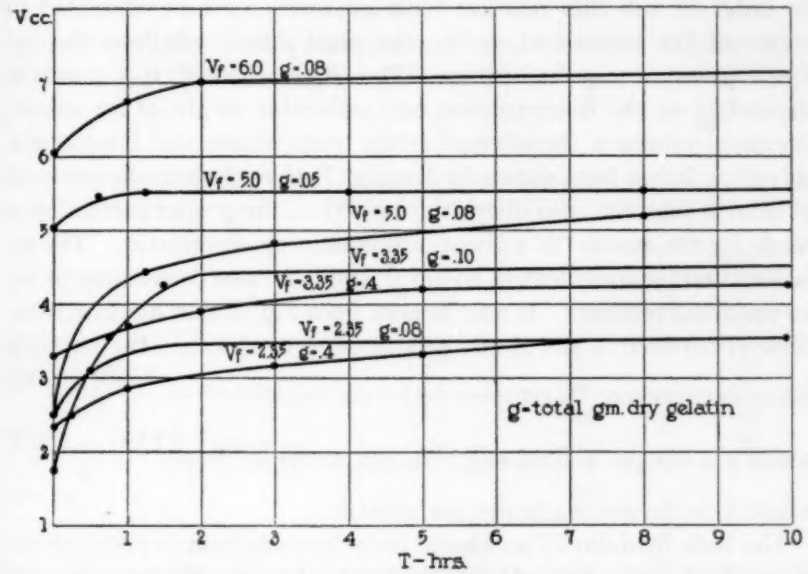

FIG. 2. Swelling of various concentrations of gelatin in water at 5°.

the osmotic pressure caused by the addition of water is therefore the same whether the water is added to the solid or liquid gelatin.

The results of some typical swelling experiments are shown in Fig. 2. It is evident that at this temperature and with thin films of gelatin, a fairly constant maximum value is reached. The experiments also show that the final value for the swelling depends only on V_f, the concentration of the gelatin at the time it solidified and, within the limits of variation used in these experiments, is independent of the concentration of the gelatin when it is put in water.

Calculation of the Equilibrium Conditions.

According to the mechanism of swelling outlined above equilibrium is reached when the osmotic pressure equals the elastic pressure. The elastic pressure in this case takes the place of the hydrostatic pressure in the case of osmosis experiments discussed in a preceding paper (6). At equilibrium then

$$OP = EP \tag{1}$$

In order to use this relation both pressures must be expressed in terms of the volume of water. In ideal dilute solutions the osmotic pressure may be written $OP = P_o/V$ where P_o is a constant depending on the concentration and molecular weight of the solute, the molal volume of the solvent and the temperature, and V is the gm. solvent. It has been shown by Kunitz (7) that the osmotic pressure of gelatin solutions also obeys this law when the proper correction is made for the amount of solvent combined with the gelatin. The relation is rather complicated, however, and it is more convenient to use an empirical relation. It may be seen from Fig. 1, that at concentrations of between .1 and .4 gm. gelatin per gm. of water, the swelling pressure curve may be represented by the equation $P = \frac{1330g - 140v}{v}$ where g is the gm. gelatin and v the gm. water or $P = \frac{1330 - 140\ V}{V}$ where V is the gm. water per gm. gelatin.

The bulk modulus of an elastic body by definition is proportional to the elastic force divided by the difference between the volume when under no strain and the volume under the force applied. Or in terms of the original volume, it is the force required to increase the volume by an amount equal to the original volume.

$$K_e = \frac{EP\ V_f}{V - V_f} \quad \text{and} \quad EP = \frac{K_e\ (V - V_f)}{V_f}$$

where V_f is the volume when under no strain and K_e is the bulk modulus. Substituting these values of OP and EP in (1)

$$\frac{1330 - 140V_e}{V_e} = \frac{K_e\ (V_e - V_f)}{V_f}$$

where V_e is the volume at equilibrium or

$$K_e = \frac{1330(1 - .105V_e)}{(V_e - V_f)} \frac{V_f}{V}$$

$$V_f = \frac{K_e V_e^2}{1330 + V_e(K_e - 140)}$$

$$V_e = \frac{(K_e - 140)V_f}{2K_e} \pm \sqrt{\frac{1330V_f}{K_e} + \left[\frac{(K_e - 140)V_f}{2K_e}\right]^2}$$

Ordinarily the total volume would be used in the calculation of the bulk modulus but since in the case of the osmotic pressure it is

TABLE I.

Swelling of Thin Layers of Gelatin on 2 mm. Glass Rods in H_2O at 5°.

V_f H_2O per gm. gelatin when cast	V_e H_2O per gm. gelatin after swelling		K_e	$\frac{K_e \times .77}{V_f}$
	Observed	Calculated $K_e = 500$		
2.35	3.5 ± .1	3.48	490	160
3.35	4.3 ± .2	4.40	550	126
5.0	5.7 ± .2	5.86	670	103
5.8	6.8 ± .3	6.56	350	46
Average.....			500	

better to use the volume of water rather than the total volume, the calculation is simplified by using the same value in the bulk modulus calculation. The use of the total volume instead of the volume of water would simply result in a different value for the bulk modulus. It may be noted that the volume of dry gelatin, however, cannot be used, as was done in an earlier paper since in that case the formula would predict that the swelling would depend only on the concentration of the block, which is not the case. The determining factor is the concentration at the time the solution solidified and not the concentration at the time it happened to be put in the water. In other words, as with any elastic body, it is necessary to define the change in volume as the change from the volume when under no elastic strain.

As will be shown later this "elastic volume" is the actual volume when the solution solidified.

A number of experiments were made as previously described and the average value of K_e calculated. The results are given in Table I. K_e is expressed in mm. mercury pressure. The values of K_e vary somewhat but the equation is of such a form that the value of this constant is very sensitive to small differences in V_e. The value is really constant for the range of gelatin used as may be seen by the fact that the values of V_e calculated by assuming a constant value for K_e are equal, within the probable error of the measurement, to the observed values. If the value of K_e be defined as the force required to increase the volume by an amount equal to the volume of the dry gelatin in the block

TABLE II.

Swelling of Gelatin in Various Forms.

$V_f = 5.8$

	On glass rods	On slides	Spheres	Thin sheets cut from cylinder
V_e	6.8	6.7	7.5	7.4
K_e	350	370	130	140
$\frac{K_e \times .77}{V_f}$	46	50	13	14

rather than the volume of water, then the value given must be multiplied by the ratio of the volume of dry gelatin to that of the water. Since 1 gm. of gelatin occupies about .77 cc., the pressure required to increase the block by an amount equal to the volume of the dry gelatin contained in it will be $K_e .77/V_f$. This value decreases in proportion to the concentration of gelatin, as would be expected. The same relation had been found by Sheppard (8) to hold approximately for the relation of modulus of elasticity to the concentration of the gelatin.

Since these experiments were made with gelatin on glass the gelatin is prevented from elongating by the glass rod. It might be expected therefore that gelatin alone would swell more. This is the case as shown by Table II which gives the equilibrium concentration for

gelatin on slides, in the form of spheres or as thin pieces cut from a cylinder. It may also be noted that the value for the bulk modulus calculated on the basis of the volume of the dry gelatin agrees with that previously calculated indirectly from separate swelling and osmotic pressure experiments (9).

It has been assumed in these calculations that the gelatin when solidified is under no elastic strain and that this concentration is therefore the determining one for the swelling. As a corollary gelatin, the concentration of which has been changed after solidifying, is under elastic strain. It might be expected then that gelatin under these

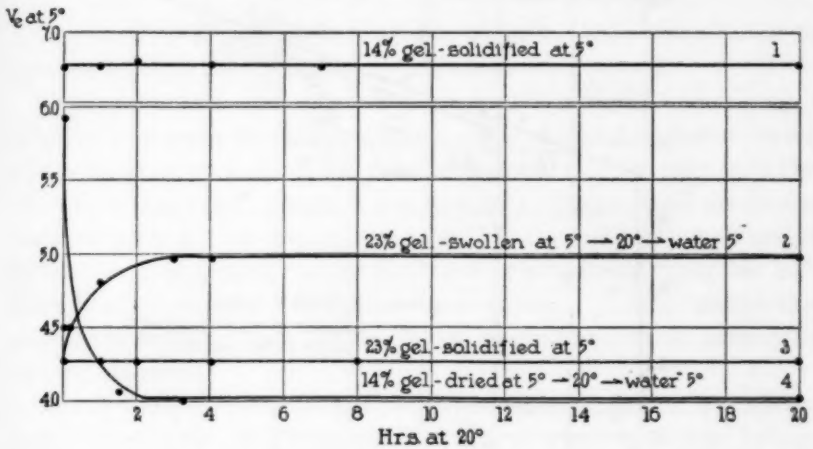

FIG. 3. Effect of time at 20° on subsequent swelling of gelatin at 5°. V_e = equilibrium volume reached on replacing in water at 5°.

conditions would show the phenomenon of fatigue in common with other elastic bodies. That this is true is shown in Fig. 3. This experiment shows the result of keeping gelatin varying lengths of time at 20° after having been swollen or dried while solid. Curves 1 and 3 show that gelatin which has been allowed to solidify at 5° undergoes no change when kept in air at 20°. The swelling which occurs on replacing in water at 5° is the same whether or not the gelatin has been kept at 20°. Gelatin which has swollen at 5° before being placed at 20°, swells further on replacing at 5°, the amount of swelling depending upon the length of time the gelatin had been at 20°.

After the first 2 hours at 20° further exposure to this temperature causes no further change in the behavior of the gelatin. The swelling now is the same as that of freshly solidified gelatin of the same concentration. Gelatin which has been partially dried shows the same behavior in that it swells much less after exposure to 20° and the swelling soon reaches a constant value. This loss of elasticity occurs very rapidly at 20° but is much slower at 5°. This is shown by Fig. 4. In this experiment, three series of rods coated with 30 per cent gelatin were allowed to swell to the equilibrium value at 5°. All but two

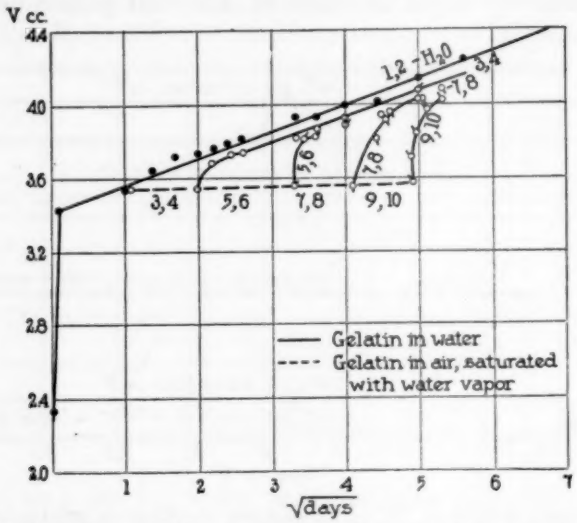

FIG. 4. Effect of time at 5° in air, after swelling, on subsequent swelling when the gelatin is replaced in water.

were then removed and placed in air at 5°. They were then returned to the water after varying periods of time. In the meantime the two rods which had been left in water continued the slow secondary swelling. On returning the other rods to water they swelled rapidly, the more so the longer the time since the beginning of the experiment, and very rapidly approached the value of those that had been in the water continuously. This shows that the secondary swelling is due to the loss of elasticity and that it is continually going on. As stated before a large block will therefore not show any maximum value since the

outside layers become fatigued and take in additional water before the internal layers have reached the equilibrium value. The same process will occur much more rapidly at a higher temperature so that even small blocks show no maximum. This secondary swelling will evidently continue until all the water has been taken up. If sufficient water is present a solution of gelatin will eventually result.

It may be noted that the secondary swelling in Fig. 4 is proportional to the square root of the time. The significance of this will be discussed under the kinetics of the process.

SUMMARY.

The swelling of isoelectric gelatin in water has been found to be in agreement with the following assumptions.

Gelatin consists of a network of insoluble material containing a solution of a more soluble substance. Water therefore enters owing to the osmotic pressure of the soluble material and thereby puts the network under elastic strain. The process continues until the elastic force is equal to the osmotic pressure. If the temperature is raised or the blocks of gelatin remain swollen over a period of time, the network loses its elasticity and more water enters. In large blocks this secondary swelling overlaps the initial process and so no maximum can be observed.

The swelling of small blocks or films of isoelectric gelatin containing from .14 to .4 gm. of dry gelatin per gm. of water is defined by the equation

$$V_e = \frac{(K_e - 140)}{2\,K_e}\,V_f \pm \sqrt{\frac{1330 V_f}{K_e} + \left[\frac{K_e - 140) V_f}{2\,K_e}\right]^2}$$

in which K_e = the bulk modulus $= \frac{PV_f}{(V_e - V_f)} = \frac{1330(1 - .105\,K_e)}{(V_e - V_f)}\,\frac{V_f}{V_e}$.

V_e = gm. water per gm. gelatin at equilibrium; V_f = gm. water per gm. gelatin when the gelatin solidified.

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THE SWELLING OF ISOELECTRIC GELATIN IN WATER.

II. KINETICS.

BY JOHN H. NORTHROP AND M. KUNITZ.

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Accepted for publication, April 27, 1927.)

Measurements of the osmotic pressure and of the swelling of gelatin in salt solutions and of the swelling pressure of gelatin have led the writers[1] to assume that gelatin was a mixture of two substances or groups of substances, one of which is soluble and the other insoluble. Solid gelatin was therefore pictured as a network of the insoluble material holding a solution of the soluble protein in its meshes. The osmotic pressure of this soluble material was assumed to be the force which caused the block to swell. According to this mechanism the swelling of gelatin should be a special case of diffusion and should be fundamentally similar to osmosis. The peculiarity of swelling lies in the fact that the block of gelatin is both membrane and solution. Swelling differs from osmosis then in that the thickness of the membrane, *i.e.* the gelatin itself, increases during the course of the experiment, and the osmotic pressure is opposed by the elasticity of the gelatin rather than by the hydrostatic pressure. It might be expected therefore that the equation for the kinetics of the process while similar to that for osmosis would differ from it in some respects. This has been found to be the case.

As pointed out in the discussion of the kinetics of osmosis, the equation is of the same form whether the water is assumed to dissolve in and diffuse through the membrane or whether it is assumed to flow through capillary pores in the membrane. The only difference lies in the physical significance of the constants. There is some evidence that the flow of water through gelatin is also through pores and since this mechanism may be more easily analysed, it has been assumed in the present paper.

[1] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1926–27, x, 161.

Formulation of the Equation.

According to Poiseuille's law the rate of flow of water through fine capillaries is proportional to the pressure times the fourth power of the radius and inversely proportional to the length of the capillary and the viscosity of the liquid. If the gelatin is assumed to consist of n pores per unit surface, then the total rate of flow of the water will be given by the expression

$$\frac{dv}{dt} = \frac{C'r^4nSP}{\eta h} \tag{1}$$

in which r is the radius of the pores, P the pressure, S the surface of the gelatin, h the length of the capillaries which is assumed to be the thickness of the gelatin, and η the viscosity of water. In order to use this expression the variable terms must be expressed as functions of v or t. In general, since the volume changes, the surface and thickness will also change. In the case of thin films of gelatin on glass, however, the surface may be assumed constant and the thickness therefore is equal to the volume divided by the surface, or $h = v/S$. The average distance traversed by the water in the case of swelling is half the thickness, so that $h/2$ must be used in place of h in equation (1).

Evaluation of P.—Since we are interested only in the amount of water which passes the outside surface of the gelatin it is not necessary to consider the pressure gradient in the gelatin, and the pressure may be assumed[2] equal to the average pressure. The pressure driving the water into the gelatin will be the difference between the swelling (osmotic) pressure which tends to cause the water to flow in and the elastic pressure of the gelatin which tends to force it out. Equilibrium is attained when these two pressures are equal. It was shown in the previous paper[3] that the swelling pressure at 5°C. from 0.14 to 0.4 gm. gelatin per gm. of water could be represented by the empirical formula

$$OP = \frac{1330 - 140V}{V},$$

[2] The validity of this assumption is discussed in the preceding paper, Northrop, J. H., *J. Gen. Physiol.*, 1926–27, x, 883.

[3] Northrop, J. H., *J. Gen. Physiol.*, 1926–27, x, 893.

where V is the volume of water per gm. of gelatin, and the elastic pressure by

$$EP = \frac{K_e(V - V_f)}{V_f},$$

where K_e is the bulk modulus and V_f is the volume when under no strain. Therefore

$$P = \frac{1330 - 140V}{V} - \frac{K_e(V - V_f)}{V_f}. \tag{2}$$

At equilibrium these two pressures are equal, *i.e.*

$$\frac{1330 - 140V_e}{V_e} = \frac{K_e(V_e - V_f)}{V_f} \quad \text{or} \quad K_e = \frac{1330(1 - .105V_e)}{(V_e - V_f)} \frac{V_f}{V_e}. \tag{3}$$

Evaluation of the Radius.—In the case of most membranes the radius of the pores would be constant, but in the case of gelatin this is probably not the case. If a block of gelatin is considered in any way analogous to a mass of separate fine particles of gelatin, then it is evident that the size of the pores which corresponds to the space between the particles will decrease rapidly as the particles swell. The exact function cannot be foretold as there is not sufficient evidence in regard to the actual structure of the gelatin. The simplest assumption, however, is that the radius of the pores between the particles would decrease as the radius of the particles increased or, approximately, $r^4 = c/V$. It will be assumed that the number of pores is constant. The size of the pores will also vary with the original concentration of the gelatin, but since this is constant for any one experiment it need not be taken into account here.

It is much more convenient to express the results in terms of the volume of water per gm. of gelatin rather than as the total volume. If g is the gm. of dry gelatin and V the volume of water per gm., then Vg equals the total volume of water, v, and $dv = gdV$.

As in the case of osmotic pressure, the equation may be solved in various ways depending upon which constants are used. The simplest expression is obtained in terms of K_e[4] the bulk modulus, V_e the equi-

[4] Since V_e may be expressed in terms of K_e and V_f it would theoretically be possible to express the equation in terms of V_f and K_e alone. Actually, however, this can only be done if a number of swelling curves are made with similar

librium volume, and V_f the volume when under no strain. In order to obtain the equation in this form equations (1) and (3) are added and the resulting equation solved for P.

Substituting these values of h, P, r^4 and v in equation (1) and simplifying and collecting the constant terms, equation (1) becomes

$$\frac{dV}{dt} = \frac{2CS^2\ 1330(bV + V_f)(V_e - V)}{V^3 g^2 V_e V_f},$$

where $b = K_e V_e/1330$; on integration, this becomes

$$C = \frac{g^2 V_e V_f}{2 \times 1330\, S^2 t}\left[\frac{V_e^2 - V^2}{2b} + \frac{(bV_e - V_f)(V_e - V)}{b^2} + \frac{2.3\ V_e^2}{(bV_e + V_f)}\right.$$
$$\left.\log\left(\frac{V_e - V_o}{V_e - V}\right) - \frac{2.3\ V_f^3}{b^2(bV_e + V_f)}\log\left(\frac{V_f + bV}{V_f + bV_o}\right)\right] \qquad (4)$$

In these experiments the last term is negligible in most cases. If K_e, the bulk modulus, is small, however, the last term becomes significant. C is the rate of flow of water under unit pressure through unit thickness and unit area of gelatin of concentration V_f. In the units used in these experiments it is cc. per hour, per sq. cm. surface, per cm. thickness per mm. mercury pressure.

In order to test this equation a number of experiments were performed with films of gelatin of various concentrations on glass slides. The gelatin was heated to 40°C., the slides weighed and then dipped in the gelatin. They were then cooled and weighed again and placed at 5°C. in stoppered tubes with wet filter paper. It was shown in the preceding paper[3] that the pressure changes for the first 3 days and then remains constant, so that in order to obtain reproducible results and also since the pressure-concentration curve is based on the value of the equilibrium pressure, the slides were usually allowed to remain at this temperature for 3 days before immersing in water. They were

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films and the results averaged before applying the equation. This is due to the fact that the value of V_e, and hence of K_e, varies slightly in individual experiments and the value of C is very sensitive to small differences in the value of V_e, especially near the end of the curve. It is therefore necessary in the calculation of individual curves such as those given to use the value of K_e and V_e determined from the particular experiment in question.

TABLE I.

Swelling of Films of Gelatin on Glass.

Experiment	S	g	V_f	V_g	t	V	$C \times 10^3$
	sq. cm.	gm.	cc.	cc.	hrs.	cc.	
2/2 *a*	25	.05	6.1	6.9	0	6.12	
					.05	6.50	1.15
					.10	6.65	1.00
					.20	6.77	.80
1/13 *a*	25	.045	6.0	6.75	0	6.0	
					.05	6.32	.63
					.10	6.48	.61
					.20	6.66	.49
2/2 *b*	25	.12	6.1	6.9	0	6.12	
					.32	6.50	1.05
					.55	6.65	1.03
					1.00	6.75	.85
c	25	.14	6.0	6.75	0	6.0	
					.2	6.27	1.22
					.4	6.45	1.30
					.8	6.62	1.30
1/24 *a*	25	.063	5.9	6.75	0	2.45	
					.10	5.0	.40
					.20	5.82	.48
					.40	6.42	.56
1/20	27	.16	5.0	5.4	0	5.0	
					.5	5.15	.140
					1.0	5.225	.123
					1.5	5.275	.120
1/24 *b*	27	.052	3.80	5.0	0	1.55	
					.05	3.3	.120
					.10	4.0	.118
					.20	4.45	.119
1/13 *b*	25	.10	3.35	4.20	0	3.35	
					.05	3.70	.360
					.10	3.82	.284
					.20	3.95	.235
12/29	42	.31	2.35	3.4	0	2.23	
					.125	2.62	.125
					.25	2.82	.122
					.50	3.0	.110

then weighed again and placed in water at 5°C. They were removed at intervals, the excess water removed with soft filter paper and weighed in air at the same temperature. This is necessary since even slight warming destroys the elasticity and greatly increases the swell-

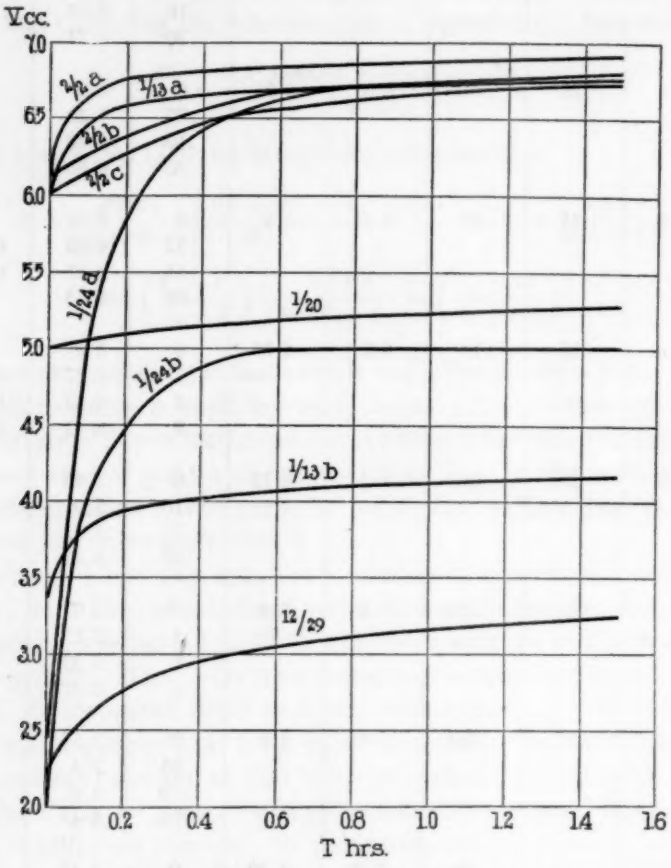

FIG. 1. Swelling films of gelatin on glass.

ing. It is also important to be sure that no change in weight has occurred before the slides and gelatin are weighed for the first time, since in the calculations it is assumed that the gelatin is of the same concentration as the solution of gelatin in which they were dipped. In some experiments the gelatin was allowed to dry partially at 5°

before placing in water. In this case the gelatin swells much more than the same concentration of gelatin which had been solidified at that concentration. It was shown in the preceding paper[3] that the equilibrium reached could be calculated by assuming that the concentration when under no strain, V_f, was the concentration when the gelatin solidified. That is, the gelatin will swell to the same final value as though it had not been dried. This is strictly true only within certain limits and provided too long a time has not elapsed after drying,

TABLE II.

Swelling of Gelatin Spheres and Plates.

13.9 per cent gelatin heated to 40° and allowed to drop slowly into 200 cc. cold toluene in 250 cc. graduate. 20 spheres used for experiment.

13.9 per cent gelatin solidified in test-tubes. 5°, 3 days. Gelatin removed by warming and sections cut.

Spheres					
s	V_f	V_s	t	V	$C \times 10^3$
gm./sphere	*cc.*	*cc.*	*hrs.*	*cc.*	
.002	6.4	7.7	0	6.4	
			1.0	6.8	.85
			2.0	7.06	.84
			4.0	7.40	.92
.005	6.4	7.7	0	6.4	
			1.5	6.8	1.02
			2.8	7.06	1.10
			7.0	7.40	0.97
Sections cut from cylinder 2.0 cm. diameter					
.037	6.3	7.5	0	6.2	
			.5	6.9	2.00
			1.0	7.13	1.75
			2.0	7.35	1.65
.10	6.3	7.5	0	6.2	
			3.0	6.9	2.45
			5.75	7.13	2.20
			11.0	7.35	2.15

as otherwise the gelatin becomes fatigued and V_f assumes a different value.

The results of some of these experiments are shown in Table I and in Fig. 1. The value of C varies slightly, but as a whole the equation appears to fit the experiment in a satisfactory way. The value of C is greater the greater the value of V_f, that is the more dilute the gelatin. This means that water flows more easily through dilute gelatin, which is a reasonable result. It can be confirmed, as will be shown

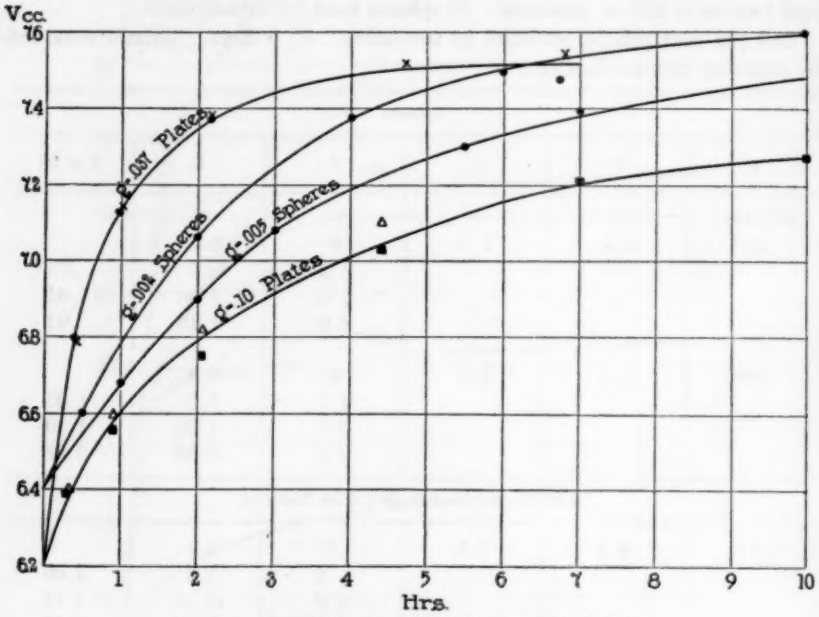

FIG. 2. Swelling of gelatin plates and spheres.

below, by direct measurement. In spite of the precautions noted some of the experiments show an anomalous course in that the gelatin swells either more or less than others of the same series. Experiment 1/20 is an example. The swelling in this case was very much less than would be expected from the average bulk modulus, and therefore the value of C although reasonably constant for this one experiment does not fit in with the other experiments. If the gelatin is placed in water immediately after solidifying, the opposite result is obtained, the gelatin swelling much more. This was ascribed to the fact that the pres-

sure is too high since it is assumed that the block is a saturated solution of one of the constituents and it requires time for the equilibrium concentration to be reached. In such cases also it was found that the value of C would be constant for that particular experiment but would differ from the other values.

Plates of Gelatin.—In the case of thin plates cut transversely from a cylinder of gelatin conditions are approximately the same as in the case just considered, since here also the greatest change in dimension is an increase in thickness and the surface may be considered constant. The results of some experiments with such plates are shown in Fig. 2 and Table II. The results have been calculated by formula (4) as before.

Spheres.—In the case of spheres the thickness instead of increasing directly as the volume increases only as the cube root of this value. The average distance which the water has to move is 1/6 the radius, which is 1/2 the ratio of volume to surface or

$$h/2 = r/6.$$

In these experiments the volume increases less than 20 per cent, so that the cube root of the volume may be considered constant for any one sphere and h assumed equal to $r_o/3$. This assumption is made in order to avoid the mathematical difficulties attendant on the integration of the fractional power of V. The surface also increases, but since the significant factor is the total number of pores and since the number presumably remains the same during any one experiment, S will also be considered a constant for any one experiment. In any case the effect of considering S variable would be within the limit of experimental error. Therefore $S = 4\pi r_o^2$ and $S/h = 12\pi r_o$, or $24\sqrt[3]{v_o} = 24\sqrt[3]{V_o g}$, approximately.

Substituting this value for S/h, equation (1) becomes

$$\frac{dV}{dt} = \frac{2 \times 1330 \times 24C\sqrt[3]{V_o g} \times (bV + V_f)(V_s - V)}{g\, V^2 V_s\, V_f};$$

or on integration:

$$C = \frac{g^{\frac{1}{3}}\, V_f V_s}{2 \times 1330 \times 24\sqrt[3]{V_o}\, t}\left[\frac{V_o - V}{b} + \frac{2.3V_s^2}{bV_s + V_f}\log\frac{V_s - V_o}{V_s - V} + \frac{2.3V_f^2}{b^2(bV_s + V_f)}\log\frac{bV + V_f}{bV_o + V_f}\right] \quad (5)$$

The last term is again negligible except when K_e, the bulk modulus, is small, *i.e.* when b is small.

TABLE III.

Swelling of Gelatin Cylinders.

Linen thread 15 cm. long dipped into melted gelatin.

$l = 15$ cm.

Experiment	s	V_f	V_e	t	V	$C \times 10^3$
	gm.	*cc.*	*cc.*	*hrs.*	*cc.*	
11/1	.08	5.80	6.90	0	5.8	
				.5	6.15	1.16
				1.0	6.37	1.15
				2.0	6.67	1.35
	.15	5.80	6.90	0	5.8	
				1.0	6.17	1.23
				2.0	6.40	1.22
				4.0	6.60	1.05
10/7	.25	6.0	7.8	0	6.0	
				2	6.5	1.40
				6	6.97	1.22
				16	7.50	1.18
11/2	.09	3.35	4.30	0	3.30	
				1.0	3.72	.170
				2.0	3.92	.162
				4.0	4.13	.154
11/2	.12	2.35	3.75	0	2.10	
				1	2.80	.150
				2	3.10	.145
				4	3.45	.145
10/13	1.0	2.35	4.5	0	2.35	
				10	3.20	.155
				20	3.60	.155
				40	4.00	.145

The results of the experiments are shown in Table II and Fig. 2. The values of C are again as constant as could be expected.

Cylinders.—The cylinders of gelatin were made by the repeated

dipping of a thread in liquid gelatin so that the gelatin on swelling did not increase in length but only in diameter. The average distance traversed by the water will be in this case $r/4$ and the total pore number Sn will equal $2\pi r_o l$, where l is the length of the cylinder. In

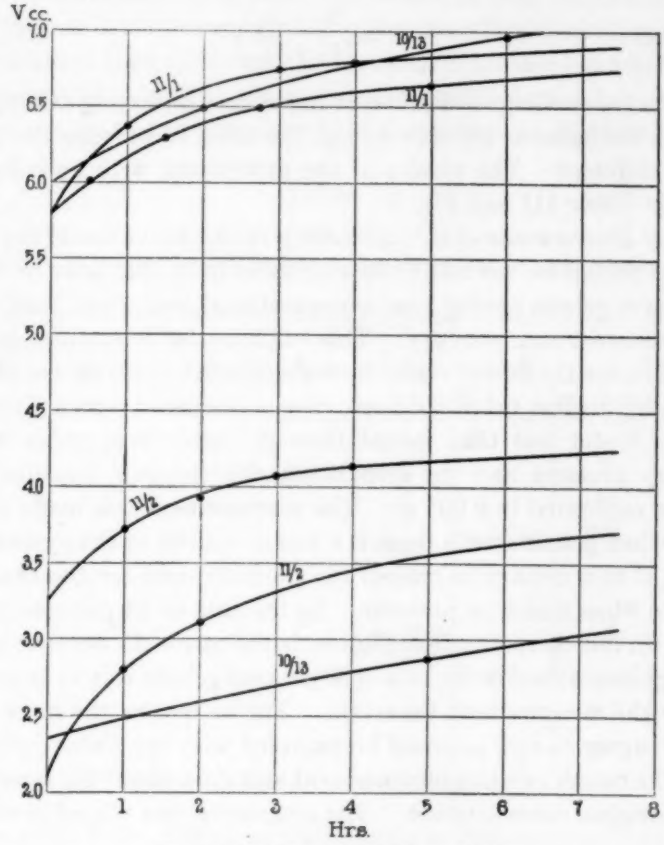

FIG. 3. Swelling of gelatin cylinders.

order to avoid fractional exponents it will be assumed again that $S = 2\pi rl$, and therefore $S/h = 4\pi l$. Substituting this value, equation (1) becomes

$$\frac{dV}{dt} = \frac{2 \times 1330 \times C \times 4\pi l(bV + V_f)(V_e - V)}{Vg \quad VV_fV_e}.$$

Integrating and collecting constant terms the equation for the rate of swelling of cylinders is therefore:

$$C = \frac{gV_fV_e}{2 \times 1330 \times 4\pi lt}\left(\frac{V_e - V}{b} + \frac{2.3V_e^2}{bV_e + V_f}\log\frac{V_e - V_e}{V_e - V} + \frac{2.3V_f^2}{b^2(bV_e + V_f)}\log\frac{V_f + bV}{V_f + bV_e}\right)$$

The effect of swelling on the rate, owing to the simplifying assumptions used, is the same as for spheres but the effect of varying the initial size is different. The results of the experiment with cylinders are given in Table III and Fig. 3.

Direct Measurement of C.—According to the derivation of the equations, C should be the rate of flow of water in cc. per hour through a cylinder of gelatin having 1 cm. cross-sectional area, 1 cm. long, under a pressure of 1 mm. mercury. This value can be determined directly by measuring the flow of water through gelatin. Gelatin was allowed to solidify in glass tubes of 0.5 cm. diameter so as to form a plug 1 cm. long.[5] Water was then forced through these plugs under 20 cm. mercury pressure and the amount passing through measured in a pipette calibrated in 0.001 cc. The measurement was made at 5°C. Since when gelatin swells there is a loss of volume of the system as a whole, it is necessary to correct the observed rate for the change in volume when under no pressure. In the case of 14 per cent gelatin ($V_f = 6$), this correction is negligible; in the case of 23 per cent gelatin it is significant; and in the case of 30 per cent gelatin it is so large as to render the measurement uncertain. The values for the more dilute gelatin agree as well as could be expected with the value calculated from the rate of swelling measurement and show about the same effect of the original concentration. The plugs were then placed in water at

[5] It may be noted in this connection that the membrane cannot be held in place by a rigid support since in that case the pressure at first presses water out of the membrane. This continues until the osmotic pressure of the membrane itself is equal to the applied pressure. In other words, no matter what concentration of gelatin is used to make the membrane, water will be removed or taken up so that when a steady filtration rate is reached the concentration of gelatin in the membrane is that which will give an osmotic pressure equal to the applied pressure.

5° for 24 hours and the measurement repeated. The rate of flow was now so slow as to be impossible to measure with any accuracy, thus verifying directly the assumption made in the beginning that the permeability decreased with the increase in swelling. In the case of the direct measurement the gelatin was prevented from expanding by the glass tube so that it is not surprising that the effect of swelling is much more marked than in the case of blocks not enclosed in a solid wall. A summary of the values of C is shown in Table IV.

The results as a whole show that the equations fit the time rate

TABLE IV.

Summary Value of C.

$C \times 10^5$ = cc. per mm. Hg pressure, per hour, per sq. cm. surface per cm. thickness.

Concentration gelatin V_f	Cylinders	Plates	Spheres	Film on glass	Average	C by direct determination	
						Not swollen	Swollen
6.0	1.15	1.7	.9	(.13)			
	1.20	2.2	1.0	1.0			
	1.30			1.0			
				1.3	1.1	1.0	<.10
				.60			
				.50			
3.35	.16			.30	.2	.5	<.10
				.12			
2.35	.15						
	.15			.12	.13		

curves quite well. Owing to the number of constants and the uncertainty of the exact value for the equilibrium volume, this agreement might be regarded as accidental. What is much more significant, in the writer's opinion, is the fact that the equations lead to a value for the rate of diffusion of water through gelatin that has been checked by direct determination and also that they express correctly the effect on the rate of swelling of varying the initial size or shape of the block. It may be noted for instance that in the case of thin films the equation

predicts that the rate of swelling per gm. of dry gelatin decreases inversely as the square of the weight of dry gelatin, in the case of spheres it decreases approximately inversely to the 2/3 power of the weight of dry gelatin, while in the case of cylinders the rate is inversely proportional to the first power of the weight of dry gelatin. If the rate of swelling is expressed simply as the total amount of water taken in per unit of time, then the equation predicts that in the case of thin films the rate is inversely proportional to the size of the block, or if the surface is constant, to the thickness. This is the result obtained in all work on swelling.

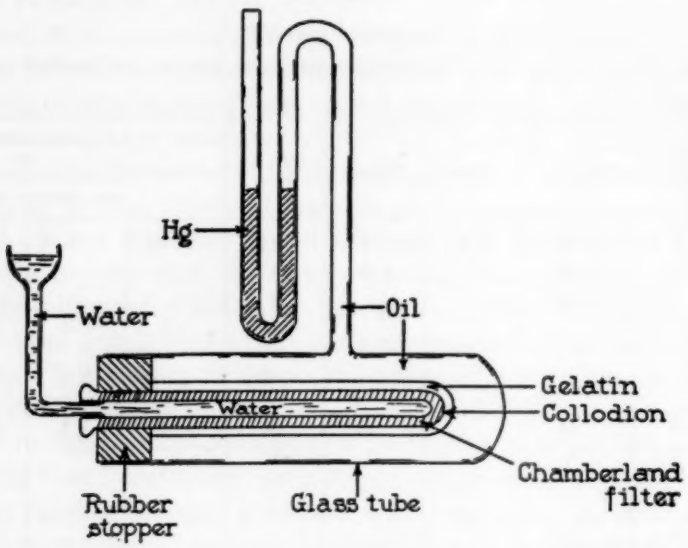

FIG. 4. Apparatus for measurement of swelling of gelatin on collodion-coated thimble.

Swelling under Experimental Conditions Which Avoid the Complicating Factors.

The preceding experiments show that the mechanism assumed for the swelling of gelatin predicts the results with considerable accuracy, but they are open to the objection that the formulas contain a number of constants and the agreement may therefore be accidental. If the mechanism is correct, however, it should be possible to predict con-

ditions under which the various secondary complications disappear or become negligible. These complications are due to the fact that the swelling pressure is opposed by the elasticity of the gelatin, a value which cannot be determined directly; and secondly to the fact that the resistance of the gelatin to the passage of the water is constantly increasing, due partly to the increase in the thickness of the layer of gelatin and partly to the decrease in the size of the pores. It was shown in the preceding paper[3] that at higher temperatures the elasticity is rapidly destroyed. It was also found that the resistance offered by collodion is very much greater than that offered by gelatin. If therefore a thin film of gelatin is coated on collodion so that the water has to pass through the collodion, the resistance offered by the gelatin will be a negligible part of the total resistance. If the experiment is carried out at 25° the elasticity of the gelatin will be partially destroyed and the term representing this force will also disappear. These conditions can be fulfilled by the apparatus shown in Fig. 4. The Chamberland filter was coated with collodion and then with gelatin. The gelatin was left at 5° for 24 hours in air and the apparatus set up as shown in a constant temperature bath at 25°C.

Under these conditions all the terms in equation (1) are constant except the pressure. The pressure will be equal to the swelling pressure minus the hydrostatic pressure. The curve for the swelling pressure of gelatin at 25° may be nearly superimposed on the curve at 5° by reducing the concentration of gelatin. The gelatin used had the same swelling pressure at 25° as 23 per cent gelatin at 5°, so that the same formula will apply for the pressure as was used for the experiments at 5° provided the concentration of gelatin be assumed to be 23 per cent. The swelling pressure is now opposed by the hydrostatic pressure just as in the osmotic pressure experiments, and the total pressure may therefore be written

$$P = OP - HP = \frac{1330 - 140V}{V} - M - Kg(V - V_o),$$

where M is the initial hydrostatic pressure and K is the mm. pressure per cc. increase in volume. Substituting this value of P, equation (1) becomes

$$\frac{dV}{dt} = \frac{CSnr^4}{h\eta g}\left[\frac{1330 - 140V}{V} - M - Kg(V - V_o)\right];$$

and bringing all the constant terms together and integrating:

$$C = \frac{2.3gV_s}{(1 + bV_s)\ 1330\ St}\left(V_s \log \frac{V_s - V_o}{V_s - V} - \frac{1}{b}\log \frac{1 + bV}{1 + bV_o}\right)$$

in which $b = KgV_s/1330$.

C in this case should be the cc. of water passing through 1 sq. cm. of the collodion-coated thimble per hour per mm. mercury pressure. When the swelling experiment was concluded the gelatin was removed and this value of C was determined directly. The results of this experiment are given in Table V and Fig. 5. The difference in the values of C for the two experiments is presumably due to differences in the membranes. The value for the second experiment is quite close to the figure determined directly. At this temperature and under these conditions the final amount of swelling is not determined by the concentration of the gelatin but depends only on the hydrostatic

TABLE V.

Swelling Gelatin on Collodion-Coated Thimble at 25°.

Experiment I. $g = 2.5$ $K = 220$ $V_s = 3.68$ $S = 103$

t	V	$C \times 10^4$
hrs.		
0	3.35	
.2	3.42	.45
.4	3.49	.51
.6	3.55	.57

Experiment II. $g = 2.0$ $K = 24$ $V_s = 5.3$ $S = 103$

t	V	$C \times 10^4$
hrs.		
0	3.35	
.4	3.78	1.05
.8	4.10	1.02
1.6	4.52	1.02
3.2	5.0	1.06

By direct determination.....1.2

pressure. This confirms the assumption made above that at 25° the elasticity of the gelatin does not enter into the equation.

Secondary Swelling.

Under the conditions adhered to in these experiments an apparent maximum is rapidly reached. This value has been called the equilibrium volume. If measurements are carried on over a long period of time, however, it will be found that there is a slow steady increase in volume. This is shown in Fig. 6, in which the swelling has been plotted

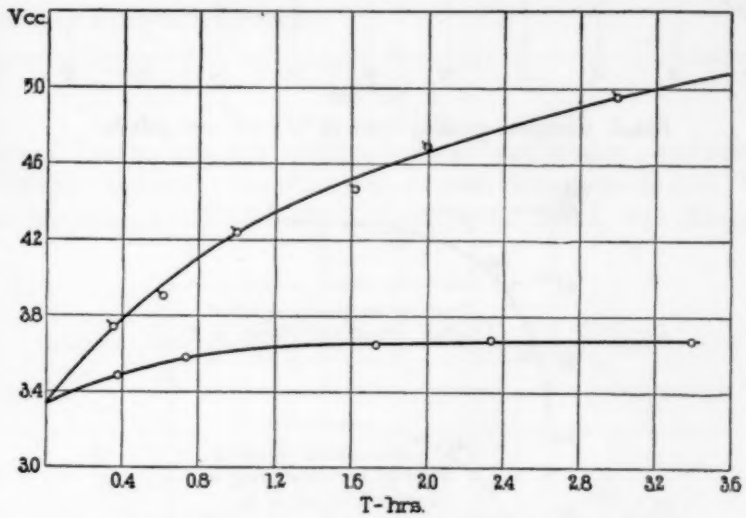

FIG. 5. Rate of swelling of gelatin on collodion-coated thimble.

against the square root of the time. This secondary swelling has been ascribed to the fatigue of the elastic force of the gelatin, and evidence was submitted in the preceding paper[3] to show that this was really the case. Fig. 6 shows that the rate of this secondary swelling is independent of the size of the block while the primary swelling is inversely proportional to the square of this quantity. This also bears out the idea that the secondary swelling is due to the fatigue of the gelatin, and hence is not regulated by the rate of diffusion of the water. This fatigue effect may be ascribed to a change in the value of V_f, which tends to approach the actual volume. It is possible to gain some idea

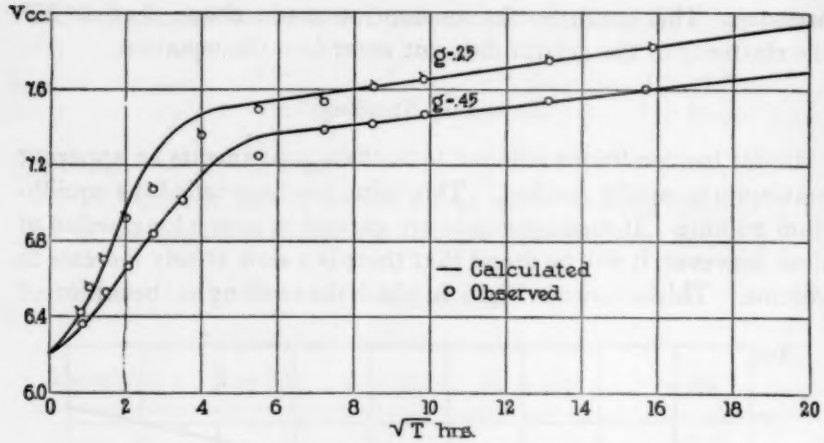

FIG. 6. Complete swelling curve of 13.5 per cent gelatin.

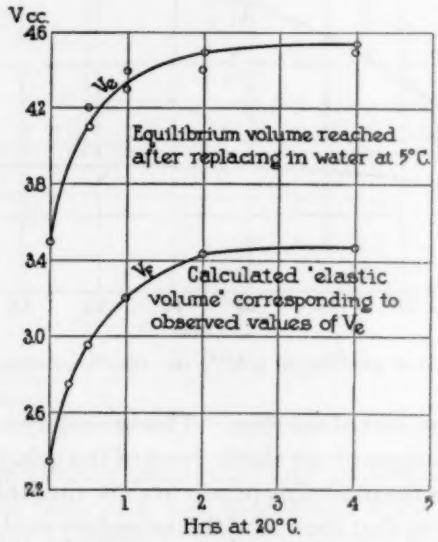

FIG. 7. Effect of time at 20° on subsequent swelling of swollen films of 30 per cent gelatin.

of the rate of change of this value by allowing swollen gelatin to remain varying lengths of time at 20° in air and then noting the swelling on returning the gelatin to water at 5°. The final volume reached in

water gives the value of V_e, and if the bulk modulus is constant the value of V_f at any time can be calculated. When these values of V_f are plotted against the time the block was kept in air, the second curve given in Fig. 7 is obtained. This curve is approximately logarithmic as might be expected and shows that the change of V_f with time is proportional to the difference between its value at time t and the equilibrium value V_{f_e}. Or

$$\frac{dV_f}{dt} = C(V_{f_e} - V_f),$$

which on integration becomes

$$C = \frac{1}{t} \log \frac{V_{f_e} - V_{f_0}}{V_{f_e} - V_f}.$$

Table VI shows that this equation holds at least as an approximation.

In this experiment the block was removed from so water that there was no change in the actual volume during the time V_f was changing,

TABLE VI.

Change of V_f with Time.

V_f–t		
t	V_f	K
hrs.		
0	2.35	
.25	2.74	.72
.50	2.98	.72
1.0	3.20	.62
4.0	3.47	

i.e. V_{f_e} was constant. In the actual experiments however, as soon as V_f changes V_{f_e} also increases. $V_{f_e} - V_f$ may therefore be considered as approximately constant, or

$$\frac{dV_f}{dt} = C', \text{ and } V_f = C't + \text{a constant.}$$

It was shown in the previous paper[3] that the equilibrium volume V_e was related to V_f by the equation $V_f = KV_e^2/1330 + V_e(K - 140)$.

The denominator of this equation may be considered constant for moderate changes in the value of V_e and the equation written

$$V_e^2 = C''V_f = C'''t; \tag{6}$$

or, since under these conditions V_e is the actual volume, $V = C\sqrt{t}$. This is the result shown in Fig. 6.

Complete Formula for Swelling.

Since the formula for the primary swelling has already been given the sum of this formula and formula (6) above will evidently represent the entire process. The equation for the primary swelling is too complicated to handle conveniently in this way, and it is necessary to omit some of the complicating factors. Since the primary swelling curve is basically logarithmic it is to be expected that it would fit the ordinary monomolecular formula provided the proper value of V_e is chosen. This of course deprives the formula of any theoretical meaning since V_e is actually determined by experiment, but may serve to give an expression for the first part of the primary swelling curve which can be used. This turns out to be the case, and it also happens that the value of V at which the secondary square root curve cuts the V axis may be used. The first part of the primary swelling may be represented therefore by the equation

$$Ct = \log \frac{V_e - V_o}{V_e - V'} \tag{7}$$

where V' is the amount of swelling due to the primary process plus the original volume, or $V' = V_e - 10^{A-Ct}$; and the secondary swelling as

$$V'' - V_e = C\sqrt{t}, \tag{8}$$

where V_e in (7) is taken arbitrarily as the value of V_e in (8) when $t = 0$.

Since V' in equation (6) is the original volume plus the increase in volume due to the primary swelling, and $V'' - V_e$ is the increase in volume due to the secondary swelling, the total volume at any time will be the sum of these quantities or

$$V = C\sqrt{t} + V_e - 10^{A-Ct}.$$

The solid lines in Fig. 6 were calculated by means of this formula and follow the general course of the actual experiment. In some of the experiments the fit was much better but there is naturally always a discrepancy near the end of the primary swelling curve.

It follows from the mechanism outlined above that if a large block is used, especially at a higher temperature, the primary swelling should be completely overshadowed by the secondary. That is, in a large block the outside layers will become fatigued and take in more water before the inside layers have swollen at all. The entire course of the

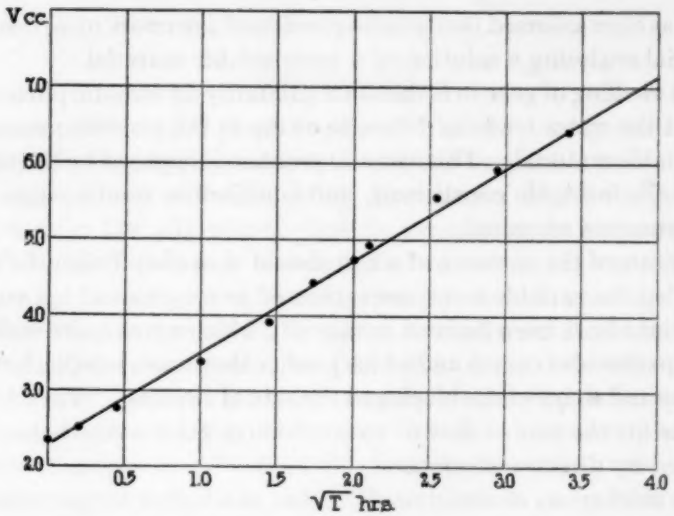

FIG. 8. Swelling of large block, 30 per cent gelatin at 27°. $g = 10$.

swelling should then be represented by the square root curve. The increase in weight of a block of 30 per cent gelatin containing about 35 cc., at 27°, is shown in Fig. 8. The process evidently follows the square root curve quite closely. According to this mechanism the gelatin should increase indefinitely or until it had taken up all the available water, and this is known to be the case.

Application to Other Types of Swelling.

If the theory of the kinetics of swelling outlined in this paper is correct it should apply in a general way to any system in which only

the solvent enters the material undergoing swelling, or in which the swelling is not affected by other substances present in solution. In the case of swelling due to a Donnan equilibrium, the pressure in the particle depends on the distribution of an electrolyte as well as on the entrance of the solvent. The theory would only apply to such systems provided conditions were such that the rate was determined by the passage of water into the solid. This condition is probably rarely if ever realized.

SUMMARY.

It has been assumed that gelatin consists of a network of an insoluble material enclosing a solution of a more soluble material.

The swelling of gelatin is therefore primarily an osmotic phenomena in that the water tends to diffuse in owing to the osmotic pressure of the soluble material. This osmotic pressure is opposed by the elasticity of the insoluble constituent, and equilibrium results when these two pressures are equal.

The rate of the entrance of water should then obey Poiseuille's law, provided the variable terms are expressed as functions of the volume. Equations have been derived in this way which agree quite well with the experimental curves and which predict the proper relation between the size and shape of the block and the rate of swelling. They lead to a value for the rate of flow of water through gelatin which has been checked by direct measurement.

The mechanism assumed predicts that at a higher temperature and under conditions such that the water has to pass through collodion before reaching the gelatin, the experiment should follow the same course as that of osmosis discussed previously. This was also found to be the case.

The slow secondary increase in swelling is ascribed to fatigue of the elastic properties of the gelatin. The rate of this secondary swelling should therefore be independent of the size of the block, in contrast to the rate of primary swelling which is inversely proportional to the size. It can further be shown that this secondary swelling should be proportional to the square root of the time, and also that with large blocks at higher temperatures the entire swelling should be of this secondary type. These predictions have also been found to be true.

ON THE NATURE OF THE DYE PENETRATING THE VACUOLE OF VALONIA FROM SOLUTIONS OF METHYLENE BLUE.

By MARIAN IRWIN.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Accepted for publication, May 12, 1927.)

I.

INTRODUCTION.

Experimental results[1] favor the theory that a basic dye penetrates a living cell very rapidly in the form of free base (which predominates at a high pH value) but so slowly in the form of salt (which predominates at low pH values) that its penetration is comparatively negligible. If this theory were correct, we should not expect a dye like methylene blue, which is very strongly basic,[2] to enter a living cell, since at the range of pH values generally available for living cells this dye exists in the form of salts. Yet methylene blue is widely known as one of the most commonly used vital stains. What is the explanation for the discrepancy between the theory presented and the observed facts? Does this indicate that the theory is inadequate, or does it mean that the dye which penetrates is not methylene blue but a less basic lower homologue, such as azure B or trimethyl thionine,[3] which is found in methylene blue solutions

[1] Overton, E., *Jahrb. wissensch. Bot.*, 1900, xliii, 669. Harvey, E. N., *J. Exp. Zool.*, 1911, x, 507. Robertson, T. B., *J. Biol. Chem.*, 1908, iv, 1. MacArthur, J. W., *Am. J. Physiol.*, 1921, lvii, 350. Irwin, M., *J. Gen. Physiol.*, 1925–27, viii, 147; 1925–26, ix, 561; 1926–27, x, 75.

[2] For discussion of the apparent dissociation constant of methylene blue *cf.* Clark, W. M., and his collaborators (Clark, W. M., Cohen, B., and Gibbs, H. D., *Pub. Health Rep., U. S. P. H., No. 23*, 1925, 1131).

[3] The apparent dissociation constant of azure B has not been determined, but we have the following reason to assume that it is a weaker base than methylene blue. In general it is found that a substance whose amino groups are completely substituted with alkyl radicles, such as tetramethyl ammonium hydroxide,

especially at a higher pH value and which is capable of existing partly in form of free base? It has been stated by some investigators[4] that lower homologues are found in samples of methylene blue, and others[5] have found that in presence of air and with an alkaline reaction methylene blue in aqueous solution is partly converted to methylene

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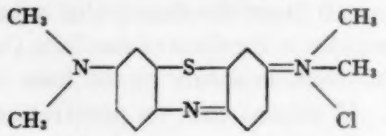
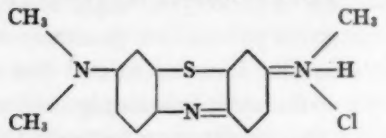
is a strong base; while a substance whose amino groups are only partially substituted by alkyl radicles, such as trimethyl ammonium hydroxide is a weaker base. Since amino groups of methylene blue or tetramethyl thionine are completely substituted with alkyl radicles, while those of azure B or trimethyl thionine are only partially substituted by alkyl radicles, as shown below, it would seem reasonable to suppose that methylene blue behaves like a strong base while azure B behaves like a weaker base.

Methylene blue or tetramethyl thionine.



Azure B or trimethyl thionine.



Difference between the chemical structure of the dye in form of free base and that of the dye in form of salt must be left undecided until further studies are made. It is uncertain as to whether such a difference between free base and salt as represented in the previous publication (Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 76) is correct. It may very well be that the salt is represented by the structure given above and that the free base is represented by a structure in which the Cl is replaced by the OH, or it is an anhydro-base.

[4] Scott, R. E., and French, R. W., *Milit. Surg.*, 1924, lv, 1. Conn, H. J., Biological stains, 1925, published by the Commission on Standardization of Biological Stains. Haynes, R., *Stain Technol.*, 1927, ii, 8. A delicate and reliable method for the detection of the presence of trimethyl thionine in methylene blue has been devised by Holmes and will appear in an early issue of *Stain Technology*. By means of this method it was found that the purest samples of methylene blue available for testing invariably contained small proportions of trimethyl thionine.

[5] Bernthsen, A., *Ann. Chem.*, 1885, ccxxx, 137. Kehrmann, F., *Ber. chem. Ges.*, 1906, xxxix, 1403. Baudisch, O., and Unna, P. G., *Dermat. Woch.*, 1919, lxviii, 4.

azure, which was found[6] to enter chloroform and to appear red. Furthermore, Kehrmann[7] has stated that methylene azure, which is a mixture of trimethyl thionine (azure B) and asymmetrical dimethyl thionine (azure A), enters substances like ether, chloroform, and benzene in form of a base and not in form of a salt, while methylene blue is not soluble in ether.

In view of the fact that the dye from methylene blue solution does not enter[8] the living cells except when the pH value of the solution is high, we may have a good reason for suspecting that the dye capable of entering a living cell is not methylene blue (in the form of a salt), but a less basic homologue (in the form of a free base), just as in the case of absorption by a substance like ether (already discussed). In this case the theory first presented would prove adequate.

One way to test this question is to study by spectrophotometric analysis the nature of the dye inside and outside the living cell. Heretofore this has not been attempted. The writer therefore proposes to give in the present paper[9] a series of spectrophotometric analyses of the dye penetrating from solutions of methylene blue into the vacuole of the living cell of marine alga *Valonia macrophysa*.

II.

Penetration of Dye into Valonia from a Solution of Methylene Blue.

Details of technique will be omitted here since they have previously been given by the writer.[10] Mention may, however, be made of several points of importance. Medium sized cells were chosen to avoid errors caused either by injury or by contamination of the sap from the stained cell wall. If too large a cell was employed, it took so long for the dye to collect in the vacuole that injury often occurred before there was a sufficient quantity of dye in the sap for spectrophotometric analysis. If too small a cell was used, the dye derived from the stained cell wall when the cell was punctured by a capillary tube for the purpose of collecting the sap exceeded the concentration of dye in the vacuole, which had

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[6] *Cf.* Baudisch, O., and Unna, P. G., Foot-note 5.

[7] *Cf.* Kehrmann, Foot-note 5.

[8] Harvey, E. N., MacArthur, J. W., and Irwin, M., see Foot-note 1.

[9] A preliminary report of these analyses has been made by the writer (Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, xxiv, 425).

[10] Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 271.

penetrated from the external dye solution before the cell was removed from the solution and punctured.

It is desirable to possess a sound basis for judging the condition of cells during the experiment. It is not a difficult matter to determine an irreversible injury, but a reversible injury is almost impossible to detect. It was therefore necessary to have a more or less arbitrary basis for judging if the cells were injured. Cells during experiment were considered to be uninjured (1) if they continued to live after they were kept in the test solution for several hours beyond the time required for experiment, (2) if they were found to be living a day or so after they had been transferred from the test solution to normal sea water, (3) if the turgidity of the cell, as detected by touch, remained the same as that of control cells (as the cells become injured they lose their turgidity).

The pH values of the sea water employed were about pH 5.5, 9.5, and 10.9. The pH value of the Bermuda sea water in which the dye was dissolved was altered and determined in the following manner. To sea water, hydrochloric acid was added until the color of the test-tube containing the sea water and brom cresol purple matched that of the standard phosphate buffer solution at pH 6 containing the same concentration of the indicator and 0.6 M sodium chloride, which roughly corresponds to the halide concentration of Bermuda[11] sea water. To sea water sodium hydroxide was added until the color of the test-tube containing the sea water and cresol phthalein matched that of the test-tube containing standard borate buffer solution at pH 9.7 or at pH 11.2, containing the same concentration of the indicator and 0.6 M sodium chloride. Owing to the slight difference in the pH value of different samples of Bermuda sea water the volume of hydrochloric acid and sodium hydroxide added varied slightly, but to 100 cc. of sea water on an average was added 1.08 cc. of 0.2 M hydrochloric acid (for pH 5.5) or 0.8 cc. of 0.2 M sodium hydroxide (for pH 9.5) or 0.45 cc. of 0.5 M sodium hydroxide (for pH 10.9).

Since the addition of sodium chloride alters the pH values of the buffer solutions, the pH values of the standard phosphate and borate buffer solutions containing 0.6 M sodium chloride were determined by means of the hydrogen electrode, and they were found to be pH 5.5 (phosphate), pH 9.5 (borate), and pH 10.9 (borate). These pH values represent only approximate pH values of the given sea water, because the colorimetric determination is not accurate for the following reason. Since in the standard buffer solutions the halide content of the sea water is represented only by sodium chloride, and since some salts are known to change the color of the indicators more than the others, the pH value of the standard buffer solution containing 0.6 M sodium chloride and that of the sea water may not be exactly the same even though the color of the test-tube containing the one matches that of the test-tube containing the other.

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[11] Bermuda sea water contains about 0.58 M halides (Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1924–25, vii, 637).

Furthermore, above pH 10.3, the magnesium in the sea water is precipitated, which in some cases interferes with colorimetric determination, and no indicators are very sensitive in this range, so that at pH 10.9 the error may not be small.

But no effort was made to avoid such sources of error since only the relative pH values were desired for these experiments. These pH values therefore are sufficiently accurate to serve the purpose in the present case, though they are approximate values.

Methylene blue is partially salted out in sea water so that two kinds of solutions were used; (1) a dye solution in which all the precipitate was allowed to remain, (2) a dye solution from which the precipitate was in greater part removed by filtering. In both cases the concentration of the dye before and after the experiment was found to remain unchanged and approximately the same results were obtained.

The following samples of methylene blue were employed, which according to the writer's knowledge represent some of the purest available.

(1) $C_{16}H_{18}N_35Cl + 1H_2O$ sent by Dr. Benda from Germany.

(2) $C_{16}H_{18}N_35Cl + 3H_2O$ sent by Dr. Benda from Germany.

(3) Sample F and Sample G, given by Dr. W. M. Clark and Dr. B. Cohen of the Hygienic Laboratory, Washington, D. C.

(4) Bleu de methylen pour Bacteriologie, Microbiologie, Physiologie, Produit, given by Dr. R. H. French of the Color Laboratory, United States Department of Agriculture, Washington, D. C.

(5) Merck's medicinal.

Owing to the difference in the solubility of different samples in sea water, different concentrations[12] varying from 0.01 to 0.04 per cent were employed.

In determining the concentration of dye in the sap the following method was used. Sap was collected by puncturing the cell (previously removed from the dye solution and wiped) by means of a sharp glass capillary tube, and by drawing up the sap from the vacuole. About 2 cc. of sap was then placed in a small test-tube and the color of this test-tube was matched with that of a test-tube of the same diameter containing a known concentration of methylene blue.

Merck's medicinal was used as the standard solution for all the samples employed because this was the only sample available in sufficient quantity to make up a series of standard solutions at different concentrations. When the concentration of dye in the sap was below 0.00004 per cent, the color appeared more greenish than the standard so that it was difficult to match the color. Furthermore, above 0.0003 per cent the color of the test-tube containing the sap appeared more purplish than that of the standard so that it again became difficult to match.

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[12] Other concentrations were used as check experiments. In all cases, if any dye entered the vacuole of uninjured cells more entered from the external solution at pH 9.5 than at 5.5, provided the experimental errors described in the text are absent.

Experiments were carried out at 25° ± 0.5°C. in an incubator with air holes through which diffused light was allowed to enter.

When living cells of *Valonia* were placed in methylene blue dissolved in sea water at these two pH values it was found that at about pH 5.5 practically no dye penetrated the vacuole, while at about pH 9.5 more entered. For example, with Merck's medicinal methylene blue, after 1 hour, at pH 9.5, the concentration of dye in the sap was about 0.00006 per cent, while at pH 5.5 it was too dilute for determination. When other samples of methylene blue, already described, were used, it was found that with some samples more dye entered than from the Merck's medicinal, while from others less entered the vacuole. But in all cases the rate of penetration of the dye into the vacuole was higher with the external dye solution at pH 9.5 than at pH 5.5. But with the samples in which the penetration was extremely slow the amount of dye found in the vacuole was so small even after several hours of exposure that unless extreme care was taken there were possibilities of experimental error arising from (1) contamination of the sap from dye in the cell wall at the time of puncturing exceeding the actual penetration of dye into the vacuole before puncturing; (2) inability to match the color of the test-tubes accurately; (3) more rapid penetration of dye due to a slight and reversible injury which cannot be detected, and which may occur if experiments are extended for several hours or if the cells at the start are not in excellent condition (the dye enters more rapidly as the cells become injured).

These sources of error might in some cases cause the rate of penetration of dye to appear the same at pH 5.5 and at pH 9.5. Furthermore, since at pH 5.5 the cells become injured more rapidly than at pH 9.5, in some cases where the injury occurred to the extent of a very slight loss of turgidity the rate of penetration at pH 5.5 was found to be higher than that at pH 9.5.

Experiments have been made with cells which have been kept in stoppered glass bottles containing some sea water for several months, as well as with cells which have been kept in a pan of sea water for several weeks. In both cases it was found that so long as the cells were not injured, the dye entered more rapidly at pH 9.5 than at pH 5.5, though in the case of the cells which have been kept in the laboratory for several months, as described above (cells appeared

less green than more recently collected cells), the dye entered more rapidly than in the case of the cells which have been kept only for a few weeks.

When the sap collected from the uninjured cells which had been exposed to the dye solution was oxidized by shaking and exposing to air with an alkaline reaction, no increase in coloration took place so that we may conclude that there was no dye in reduced form present in the sap.

When the pH value of the sap was determined after the living cells of *Valonia* had been exposed to sea water at pH 5.5, 9.5, and 10.9 for 4 hours, no change in the pH value of the sap occurred if the cells were not injured.

III.

Spectrophotometric Analysis.

The nature of the dye in the external solution and in the vacuolar sap of uninjured and injured cells was tested by means of spectrophotometric determinations. The measurements were made at the Color Laboratory in Washington, D. C., by W. C. Holmes, of whose collaboration the writer desires to express her appreciation.

The instrument employed was a Hilger wave-length spectrometer, equipped with a Nutting photometer. Either 1 or 2 cm. layers of solution were examined, depending on the concentration of dye in the solutions and the quantities of solution available. The measurements were carried out over the spectral range between 540 and 690 mμ. The concentration of dye was adjusted, insofar as was possible, to afford maximum visual sensitivity at and near the absorption maximum of the dye in dilute aqueous solution. All recorded values in this restricted region are averages of a considerable number of measurements.

A brief statement of spectroscopic criteria is advisable at this point. The absorption maximum of methylene blue in dilute aqueous solution is approximately 665 mμ. The corresponding maximum of trimethyl thionine is approximately 650 mμ. Although the average visual sensitivity in this region of the spectrum is relatively inferior it is readily possible to locate absorption maxima (with favorable dye concentrations) within a possible variation of about $\pm$1mμ.

The determination of the approximate absorption maximum, accordingly, differentiates the two dyes with absolute certainty. It affords, moreover, a reliable, if somewhat rough, criterion of the relative proportions of the dyes in question when both are present. Owing to the relatively limited spectral interval between their bands the band of a mixture of the dyes does not inhibit the individual maxima of its two component bands, but, rather, a single composite maximum of which the location varies with dye proportions. The absorption maximum of a mixture containing 66 per cent of methylene blue and 33 per cent of trimethyl thionine, for example, falls at approximately 660 mμ, while that of a mixture of 33 per cent of methylene blue and 66 per cent of trimethyl thionine falls at approximately 655 mμ.

It may be noted that the employment of suitable spectrophotometric ratios would afford a more exact definition of relative dye proportions. The basic data requisite for this procedure, however, were not available when the present investigation was begun and it was felt that the mere determination of the approximate absorption maxima of solutions would afford ample evidence of their character for present purposes.

Both methylene blue and trimethyl thionine exhibit secondary absorption in the general spectral region near 600 mμ. Both dyes are held to exist in aqueous solutions in a state of tautomerism between two dye forms.[13] In the present investigation considerable variations were noted in the apparent tautomeric equilibria between dye forms. These arise primarily from variations in dye concentration and are also influenced by other factors. A discussion of these phenomena is unnecessary in this paper. It is sufficient to note that their occurrence does not modify in any appreciable degree the relative absorption of the dyes at different wave-lengths within the critical spectral region between 650 and 665 mμ, or invalidate conclusions derived from variations in absorption within that region.

When such analyses were made some very interesting facts were obtained, as follows:

I. A sample of methylene blue,[14] dissolved in (1) Bermuda sea

[13] Holmes, W. C., *Ind. and Eng. Chem.*, 1924, xvi, 35; *Stain Technol.*, 1926, i, 17.

[14] Several samples were employed (see Section II in text).

water, (2) sap of *Valonia macrophysa*, and (3) artificial sap,[15] gave absorption maxima[16] characteristic of methylene blue, about 665 mμ (see Table I and Fig. 1, Curves *A*, *B*, and *C*).

II. The dye allowed to diffuse out of the cell wall (which had been previously stained by placing the living cells for a few minutes in sea water containing methylene blue), into artificial sap of *Valonia* gave

TABLE I.

Solutions	Primary absorption maximum
	mμ
Methylene blue dissolved in sea water	665
Methylene blue dissolved in the sap of *Valonia*	665
Methylene blue dissolved in artificial sap of *Valonia*	665
Dye which has diffused out of the cell wall into artificial sap, after the cell wall of living cells of *Valonia* has been stained in methylene blue (dissolved in sea water)	665
Dye found in sap from the vacuole of injured cells of *Valonia* when cells were stained in methylene blue dissolved in sea water at pH 9.5	663
Dye found in sap from the vacuole of injured cells of *Valonia* when cells were stained in methylene blue dissolved in sea water at pH 5.5	665
Trimethyl thionine dissolved in sap of *Valonia*	650
Dye found in the vacuole of uninjured cells of *Valonia* when cells were stained in methylene blue dissolved in sea water at pH 9.5	650
Dye absorbed by chloroform from methylene blue dissolved in sea water at pH 9.5: This was freed from chloroform by absorbing it in distilled water	650
Dye absorbed by chloroform from methylene blue dissolved in sea water at pH 5.5: This was freed from chloroform by absorbing it in distilled water	655
Methylene blue dissolved in distilled water	665
Dye absorbed by chloroform from methylene blue dissolved in M/150 buffer mixtures at pH 5.5 or at pH 9.5: This was freed from chloroform by absorbing it in distilled water	650

the absorption maximum of 665 mμ characteristic of methylene blue (Table I and Fig. 1, Curve *D*).

[15] The pH value of the sap is about 5.8. The sap contains about 0.6 M halides (*cf.* Osterhout, W. J. V., and Dorcas, M. J., Foot-note 11).

[16] The absorption curve thus obtained resembles that of a higher concentration of methylene blue dissolved in distilled water. This may be due to the effect of salt on the dye, as suggested by Dr. W. C. Holmes.

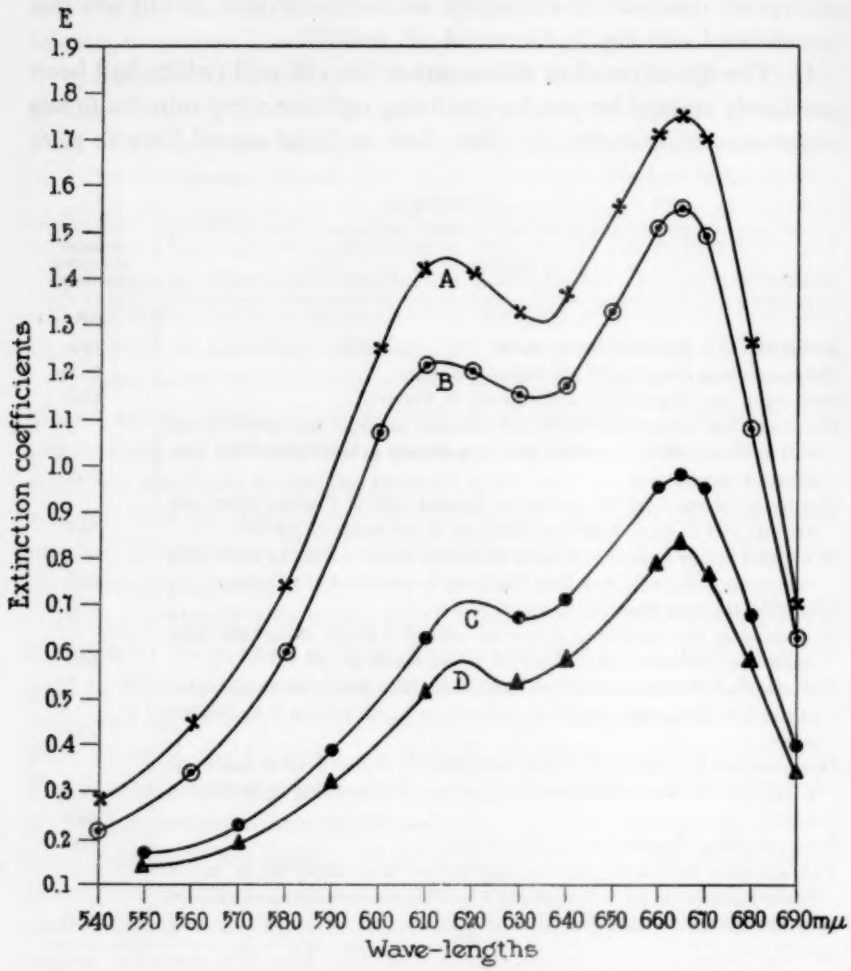

FIG. 1. Extinction coefficients are plotted as the ordinates and the wave-lengths as the abscissæ. Curve *A* represents the methylene blue dissolved in sea water, Curve *B* in the sap of *Valonia*, Curve *C* in the artificial sap of *Valonia*, Curve *D* the dye that has diffused from the cell wall into artificial sap after the cell wall of living cells of *Valonia* has been stained in methylene blue dissolved in sea water.

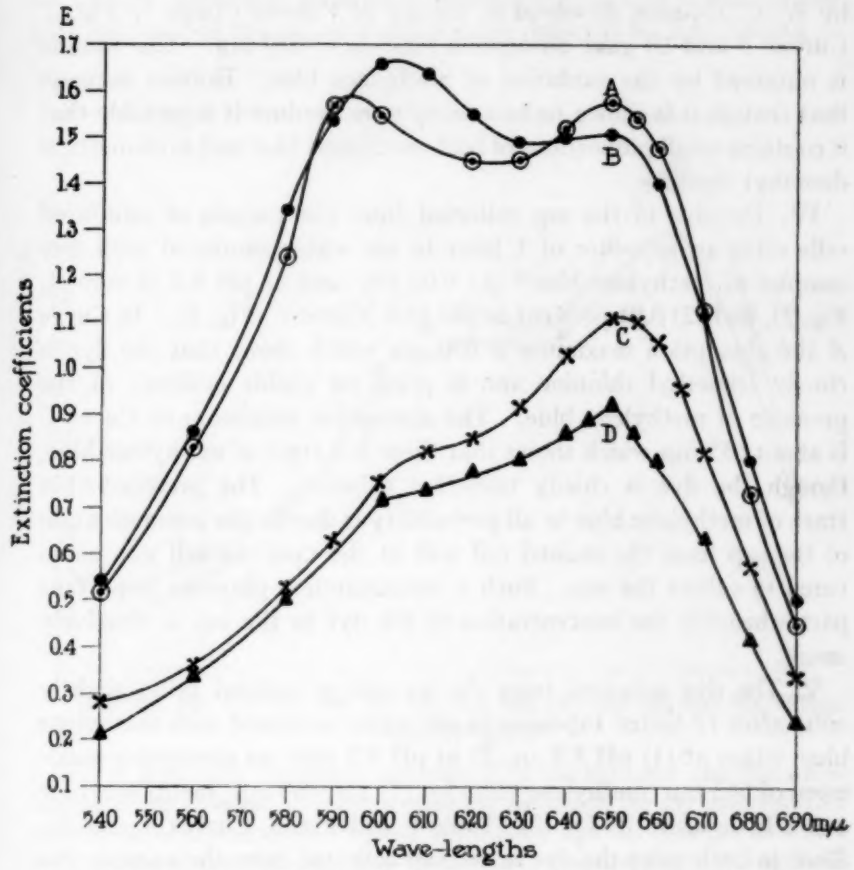

FIG. 2. Extinction coefficients are plotted as the ordinates and the wave-lengths as the abscissæ. Curves *A* and *C* (symbols ⊙ and ×) represent the dye which was found in the vacuolar sap of uninjured cells of *Valonia* after the cells have been stained for 1 hour in one sample of methylene blue dissolved in sea water at pH 9.5 (Curve *A*) and in another purer sample at pH 10.9 (Curve *C*). Curves *B* and *D* represent the sample of trimethyl thionine or azure B dissolved in sap of *Valonia* at two dilutions, corresponding approximately to two dilutions represented by Curves *A* and *C* respectively. Curve *B* corresponds with Curve *A* and Curve *C* with Curve *D*.

III. Two concentrations of trimethyl thionine or azure B (sent by W. C. Holmes) dissolved in the sap of *Valonia* (Table I, Fig. 2, Curves *B* and *D*) gave absorption maxima of 650 mμ. This sample is obtained by the oxidation of methylene blue. Holmes suggests that though it is shown to be a fairly pure product it is possible that it contains small proportions of both methylene blue and asymmetrical dimethyl thionine.

IV. The dye in the sap collected from the vacuole of uninjured cells after an exposure of 1 hour to sea water saturated with two samples of methylene blue[17] (1) 0.04 per cent at pH 9.5 (Curve *A*, Fig. 2), and (2) 0.01 per cent at pH 10.9 (Curve *C*, Fig. 2). In Curve *A* the absorption maximum is 650 mμ which shows that the dye is chiefly trimethyl thionine and it gives no visible evidence of the presence of methylene blue. The absorption maximum of Curve *C* is about 652 mμ which shows that there is a trace of methylene blue, though the dye is chiefly trimethyl thionine. The presence of a trace of methylene blue in all probability is due to the contamination of the sap from the stained cell wall at the time the cell was punctured to collect the sap. Such a contamination plays an important part whenever the concentration of the dye in the sap is relatively small.

V. The dye collected from the vacuole of injured cells (slightly soft) after 12 hours' exposure to sea water saturated with methylene blue, either at (1) pH 5.5, or (2) at pH 9.5 gave an absorption maximum of 665 mμ (methylene blue) for (1) and 663 mμ (methylene blue and a little azure B) for (2) (Table I, and Fig. 3, Curves *A* and *B*). Since in both cases the dye in the sap collected from the vacuole was diluted with the sap collected from the vacuole of unexposed living cells, the heights of the curves which vary with dilution (the higher curve corresponding to the higher concentration) given in Fig. 3 do not show true relative concentrations of the dye found in the vacuole.

The azure B found by spectrophotometric analysis in the sap collected from the vacuole of uninjured cells of *Valonia* is not due to the transformation of methylene blue into azure B after methylene blue

[17] Samples employed are specified in the text in Section II. Owing to the fact that the purpose of these experiments is not to determine the purity of these samples, the sample used for each result is not specified.

has penetrated from the external dye solution into the vacuole because not enough conversion takes place during 1 to 3 hours in the methylene blue dissolved in the sap of *Valonia* to be detected by this method.

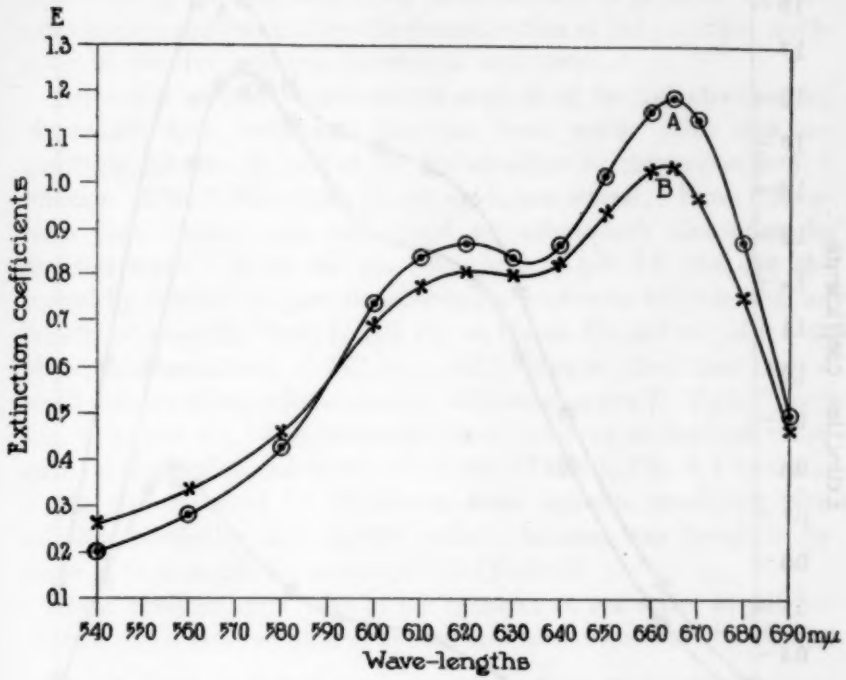

FIG. 3. Extinction coefficients are plotted as the ordinates and the wave-lengths as the abscissæ. Curves representing the dye in the sap collected from the vacuole of injured cells of *Valonia* and diluted with sap after the cells have been stained in the methylene blue dissolved in sea water, Curve *A* at pH 5.5 and Curve *B* at pH 9.5.

IV.

Absorption of Dye by Chloroform from Methylene Blue Solution.

In view of the fact that a similarity was found between *Valonia* and chloroform in their behavior toward other basic[18] dyes, in that

[18] When living cells of *Valonia* were placed in different basic dyes, Lauth's violet, neutral red, and brilliant cresyl blue, it was found that the higher the pH values of the dye (*viz.* between pH 5 and pH 8), the more rapidly the dye

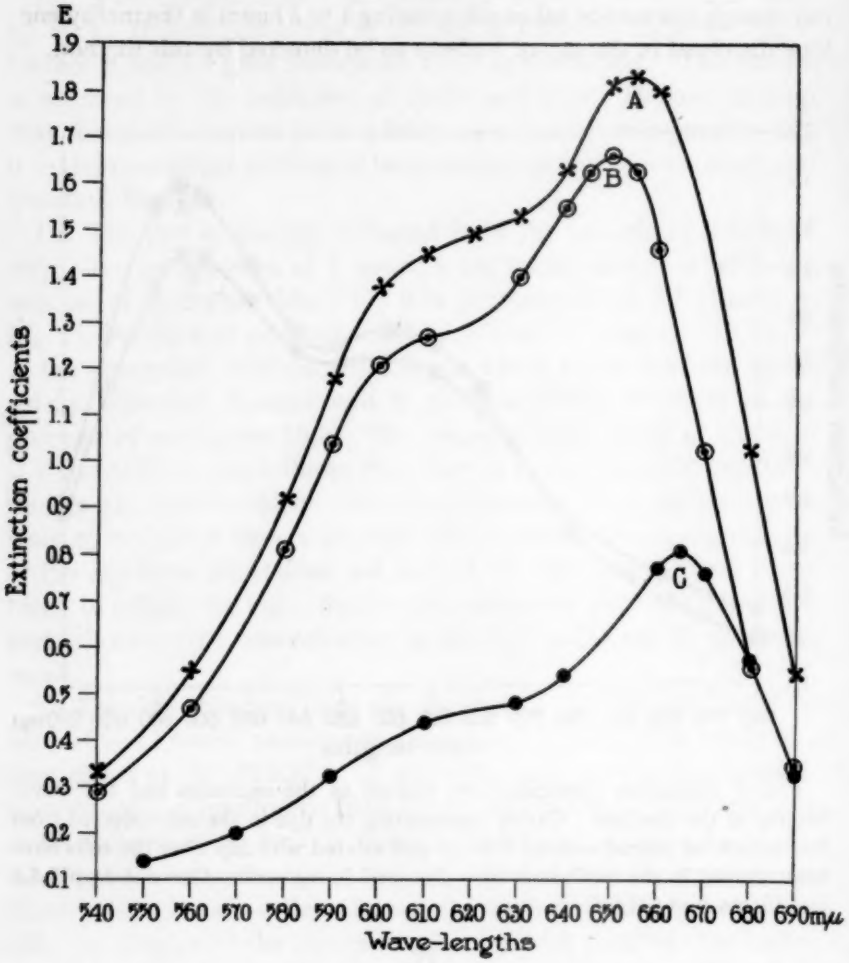

FIG. 4. Extinction coefficients are plotted as the ordinates and the wave-lengths as the abscissæ. Curves *A* and *B* represent the dye which was absorbed by chloroform from methylene blue dissolved in sea water, Curve *A* at pH 5.5 and Curve *B* at pH 9. In both cases the dye was freed from chloroform by its subsequent absorption in distilled water. Curve *C* represents methylene blue dissolved in distilled water.

both take up the dye in the form of free base much more readily than in the form of salt, it seemed possible that chloroform, like *Valonia*, takes up chiefly trimethyl thionine or azure B from a solution of methylene blue, which may be determined by means of spectrophotometric analysis and by the determination of the partition coefficient of the dye between chloroform and water.

Heretofore no spectrophotometric analysis of the dye absorbed by chloroform from methylene blue has been made. This was accordingly done in the case of the dye absorbed by chloroform from a solution of methylene blue (made up in sea water). From chloroform thus stained, dye was freed by subsequent absorption in distilled water. When the sea water was at pH 9.5, the dye absorbed by chloroform gave the absorption maximum 650 mμ, characteristic of azure B (Table I, and Fig. 4, Curve *B*), and at pH 5.5 an absorption maximum of 655 mμ (which showed that there was a small amount of methylene blue in addition to azure B, Table I, and Fig. 4, Curve *A*). The methylene blue dissolved in distilled water gave an absorption maximum of 665 mμ (Table I, Fig. 4, Curve *C*).

The dye absorbed by chloroform from aqueous methylene blue solution (made up with M/150 buffer mixtures) was found to be azure B, both at pH 5.5 and at pH 9.5 (Table I).

These analyses show that in the presence of sea water at pH 5.5 azure B and a small amount of methylene blue are absorbed by chloro-

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entered the vacuole. Relative rate of penetration differed with various basic dyes. Such differences in the rates corresponded roughly with the differences in the degree of absorption of these dyes by chloroform at different pH values of the sea water, and in the basicity of the dyes. When the amount of brilliant cresyl blue absorbed by chloroform or by *Valonia* is plotted against the external pH values, an S-shaped curve is obtained in both cases.

In view of the fact that in presence of sea water a basic dye in form of salt, as well as in form of free base, enters the chloroform, it is difficult to obtain with any accuracy the distribution coefficient of the dye only in form of free base between chloroform and sea water. This complication, however, was absent in the case of the basic dyes dissolved in M/150 buffer solutions, so that it was possible to make a comparison on a quantitative basis between the absorption of dye in form of free base by the vacuole of *Nitella* and by chloroform (Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 561).

form, while from sea water at pH 9.5 and from dilute buffer solutions at both pH values chiefly azure B is absorbed.

As to the experiments on the distribution of the dye between methylene blue dissolved in sea water and chloroform the following results were obtained.

When 3 cc. of chloroform were shaken up with 10 cc. of 0.002 per cent methylene blue dissolved in sea water at pH 9.5 and pH 5.5, it was found that the apparent partition coefficient of the dye between chloroform and sea water at pH 9.5 was about 2 (*i.e.* more concentrated in chloroform), while at pH 5.5 it was about 1.3. These might be called the apparent partition coefficients, since they merely represent the ratio of the concentration of the dye in the chloroform to that of the dye in sea water, without taking into account whether the dye is azure B, methylene blue, or a mixture of both.

The color of the dye in the chloroform when absorbed from methylene blue in sea water at pH 9.5 is reddish purple and from methylene blue in sea water at pH 5.5 is blue. Since the dry salt of methylene blue and that of azure B dissolved in chloroform is blue, the dye in the chloroform absorbed from methylene blue in sea water at pH 5.5 may possibly represent a mixture of methylene blue and azure B, in form of salt, while the dye in chloroform absorbed from methylene blue in sea water at pH 9.5 is azure B chiefly in form of free base. These experiments are insufficient to show whether or not these dyes in form of salt enter chloroform as undissociated molecules which may possibly be formed to a certain extent in presence of so high a concentration of salt such as sodium chloride in the sea water.

Just as in the case of the vacuole of uninjured cells of *Valonia* the dye which is taken up by chloroform from methylene blue in sea water at pH 9.5 is chiefly azure B in form of free base and not methylene blue.

Whether methylene blue is capable of penetrating the vacuole from methylene blue in sea water at pH 5.5 cannot be determined, since the dye does not penetrate in sufficient quantity for spectrophotometric analysis. Since the concentration of undissociated molecules of methylene blue in form of salt possibly present in sea water is not determined, this result neither proves nor disproves the theory that the undissociated molecules enter the vacuole of uninjured cells more rapidly than the ions.

It is not certain as to whether methylene blue enters the chloroform in constant amount at all pH values or only at lower pH values. Further experiments are necessary to determine this point.

V.

Penetration of Azure B into the Vacuole of Valonia and into Chloroform.

If our supposition is correct that azure B is a weaker base than methylene blue, and capable of existing in form of free base at higher pH values, then, according to the theory presented in Section I, a pure sample of azure B should penetrate into the vacuole of *Valonia* and into chloroform more when the pH value of the sea water is higher.

Living cells of *Valonia* were therefore placed for ½ hour in 0.04 per cent azure B (1) made by Holmes, (2) extracted by chloroform from methylene blue solution (made up with borate buffer at pH 9.5). With both samples approximately the same results were obtained, in that at pH 9.5 the rate of penetration was much higher (about 0.001 per cent dye in sap) than at pH 5.5 (dye in sap was too dilute for accurate determination).

When 3 cc. of chloroform were shaken up with 10 cc. of the azure B dissolved in sea water at pH 9.5 and at pH 5.5, the partition coefficient of the azure B (made by Holmes) between chloroform and sea water at pH 9.5 was 14.9 and at pH 5.5 was 1.8 (*i.e.* the dye was more soluble in chloroform than in sea water).

The color of the dye in chloroform when sea water was at pH 9.5 was reddish purple, while it was blue at pH 5.5.

Since the dry salt of azure B dissolved in chloroform appears blue, the azure B taken up by chloroform from sea water at pH 5.5 may be in form of salt, and at pH 9.5 in form of free base (which is reddish purple).

VI.

DISCUSSION.

From these results we may conclude that the vacuole of uninjured cells of *Valonia macrophysa* takes up chiefly trimethyl thionine (azure B) from the solution of methylene blue which contains so little azure B (as impurity) that it cannot be detected by the spectro-

photometer. As soon as cells are injured methylene blue enters. The writer's results and conclusion are contrary to those obtained by M. M. Brooks,[19] who states that the vacuole of uninjured cells of *Valonia macrophysa* takes up dye from methylene blue solution with the same speed at all pH values (from pH 5 to 9), from which she concludes (without analysis of the dye in the vacuole) that methylene blue (in form of salt) enters the vacuole of uninjured cells.

The writer's experiments show that the penetration of dye into the vacuole of uninjured cells from a solution of methylene blue does not discredit the theory that the basic dye enters the vacuole chiefly in the form of free base, since the dye which penetrates is found to be chiefly a lower homologue of methylene blue, azure B, which is less basic and capable of existing in part in the form of free base at higher pH values. Azure B behaves like all other basic dyes in that its relative rate of penetration depends on the amount of dye in form of free base present, which corresponds with the pH value of the external solution (the higher the pH value the more dye is in form of free base and the more rapid is the rate of penetration). That this difference in the rate of penetration at varying pH values is not due primarily to the effect of different pH values on the protoplasm is shown by the fact that the relative rates of penetration at a given series of pH values differ with different basic dyes.

The writer's previous statement that the vacuole of living cells, such as that of *Valonia*, behaves very much like chloroform toward basic dyes, in that they both take up the dye in the form of free base, is still further supported by the fact that they both take up primarily azure B from methylene blue solution at higher pH value.

Undoubtedly the penetration of a basic dye depends chiefly on two factors under such experimental conditions, (1) on the apparent dissociation constant of the dye, (2) on the partition coefficient of the dye between the vacuolar sap and the external solution, and in some cases on that of the dye between the vacuolar sap and the protoplasm. In case there is a combination of dye with some constituent of the sap, this factor must be brought into consideration. With chloroform also penetration depends on the dissociation constant and the partition coefficient.

[19] Brooks, M. M., *Am. J. Physiol.*, 1926, lxxvi, 360.

There is a similarity between chloroform and the vacuole of uninjured cells of *Valonia* in that they are both capable of taking up azure B and some other basic dyes in form of free base, but in certain cases chloroform and *Valonia* are found not to behave alike. For example, some acid dyes are slightly soluble in chloroform but they do not penetrate the vacuole of uninjured cells of *Valonia*. However such an analogy is not complete since the ability of the dye to collect in the vacuole may not only depend on the ability of the protoplasmic layer (between protoplasm and external solution, or between protoplasm and the vacuole) to absorb the dye but also on its power to give up the dye. Experiments are being done with this consideration in view.

The fact that azure B instead of methylene blue is found in the vacuole is not proof that methylene blue does not enter the protoplasm. It might enter the protoplasm though it does not penetrate into the vacuole. One way to arrive at a definite conclusion is to determine the nature of the dye inside the protoplasm, after the dye has been allowed to penetrate the cell in uninjured condition; but with the protoplasmic layer of *Valonia* this cannot be accomplished since it cannot be removed for examination without contamination or injury. Furthermore there is no way of determining whether or not methylene blue enters protoplasm and is converted to azure B or trimethyl thionine.

These experiments show the danger of drawing conclusions as to permeability or as to oxidation-reduction potentials from the experiments on the penetration of dye from a solution of methylene blue into living cells, unless we know the nature of the dye both in the external solution and inside the cells.

These experiments were repeated with *Nitella* and gave approximately the same results.

The writer wishes to thank Miss Helen McNamara for her faithful assistance in carrying out the experiments.

SUMMARY.

When uninjured cells of *Valonia* are placed in methylene blue dissolved in sea water it is found, after 1 to 3 hours, that at pH 5.5

practically no dye penetrates, while at pH 9.5 more enters the vacuole. As the cells become injured more dye enters at pH 5.5, as well as at pH 9.5.

No dye in reduced form is found in the sap of uninjured cells exposed from 1 to 3 hours to methylene blue in sea water at both pH values.

When uninjured cells are placed in azure B solution, the rate of penetration of dye into the vacuole is found to increase with the rise in the pH value of the external dye solution.

The partition coefficient of the dye between chloroform and sea water is higher at pH 9.5 than at pH 5.5 with both methylene blue and azure B. The color of the dye in chloroform absorbed from methylene blue or from azure B in sea water at pH 5.5 is blue, while it is reddish purple when absorbed from methylene blue and azure B at pH 9.5. Dry salt of methylene blue and azure B dissolved in chloroform appears blue.

It is shown that chiefly azure B in form of free base is absorbed by chloroform from methylene blue or azure B dissolved in sea water at pH 9.5, but possibly a mixture of methylene blue and azure B in form of salt is absorbed from methylene blue at pH 5.5, and azure B in form of salt is absorbed from azure B in sea water at pH 5.5.

Spectrophotometric analysis of the dye shows the following facts.

1. The dye which is absorbed by the cell wall from methylene blue solution is found to be chiefly methylene blue.

2. The dye which has penetrated from methylene blue solution into the vacuole of uninjured cells is found to be azure B or trimethyl thionine, a small amount of which may be present in a solution of methylene blue especially at a high pH value.

3. The dye which has penetrated from methylene blue solution into the vacuole of injured cells is either methylene blue or a mixture of methylene blue and azure B.

4. The dye which is absorbed by chloroform from methylene blue dissolved in sea water is also found to be azure B, when the pH value of the sea water is at 9.5, but it consists of azure B and to a less extent of methylene blue when the pH value is at 5.5.

5. Methylene blue employed for these experiments, when dissolved

in sea water, in sap of *Valonia*, or in artificial sap, gives absorption maxima characteristic of methylene blue.

Azure B found in the sap collected from the vacuole cannot be due to the transformation of methylene blue into this dye after methylene blue has penetrated into the vacuole from the external solution because no such transformation detectable by this method is found to take place within 3 hours after dissolving methylene blue in the sap of *Valonia*.

These experiments indicate that the penetration of dye into the vacuole from methylene blue solution represents a diffusion of azure B in the form of free base. This result agrees with the theory that a basic dye penetrates the vacuole of living cells chiefly in the form of free base and only very slightly in the form of salt. But as soon as the cells are injured the methylene blue (in form of salt) enters the vacuole.

It is suggested that these experiments do not show that methylene blue does not enter the protoplasm, but they point out the danger of basing any theoretical conclusion as to permeability on oxidation-reduction potential of living cells from experiments made or the penetration of dye from methylene blue solution into the vacuole, without determining the nature of the dye inside and outside the cell.

UDDER SIZE IN RELATION TO MILK SECRETION.

By JOHN W. GOWEN AND ELMER R. TOBEY.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J., and the Maine Agricultural Experiment Station, Orono.)

(Accepted for publication, May 31, 1927.)

Our knowledge of the mammary gland as related to its size and function has been largely based on the observational relationship which exists between udder size and the milk the cow is able to give.[1] Briefly considered the data available consist of two measurements, the milk production of the cow in pounds and the relative degree of perfection of the udder in size, shape, and quality as measured on an arbitrary scale called her score. Study of this information on 1674 Jersey cattle shows a correlation between the size and quality of the udder and the milk yield which the cow was able to produce. This correlation, while markedly significant, is low, .19 ± .016. The evidence thus points to the conclusion that despite the obvious sources of error the size of the udder is a function at least of the milk which the cow is able to secrete. The just published work of Gaines and Sanmann[2] supports this hypothesis while the work of Maxwell and Rothera[3] and the opinions of many dairymen and veterinarians citing the size of the udder as being too small to hold the milk the

[1] Gowen, J. W., Conformation and its relation to milk-producing capacity in Jersey cattle, *J. Dairy Sc.*, 1920, iii, 1. Studies on conformation in relation to milk-producing capacity in cattle. II. The personal equation of the cattle judge, *J. Dairy Sc.*, 1921, iv, 359. Studies on conformation in relation to milk-producing capacity in cattle. III. Conformation and milk yield in the light of the personal equation of the dairy cattle judge, *Annual Rep. Maine Agric. Exp. Station*, 1923, 69.

[2] Gaines, W. L., and Sanmann, F. P., The quantity of milk present in the udder of the cow at milking time, *Am. J. Physiol.*, 1927, lxxx, 691.

[3] Maxwell, A. L. I., and Rothera, A. C. H., The action of pituitrin on the secretion of milk, *J. Physiol.*, 1914–15 xlix, 483.

cow is able to give at any one time controvert it.[4] Stated quantitatively the problems before us are: (*a*) what proportion of the milk given by the cow at milking is already stored in the udder; (*b*) what correlation exists between udder size and milk yield; (*c*) how much secreting tissue is necessary to manufacture a pound of milk; (*d*) what is the relation between secreting and supporting tissue in the udder.

For the work herein cited nine dairy cows were used. These cows were milked twice a day, the time of the first milking being 1.00 p.m., and that of the second milking, 10.00 p.m. The cows were milked on these hours for 5 days before they were killed. Their milk was weighed after each milking and a sample of the milk taken for the analysis of the lactose content. The cows were then shipped 10 miles to a slaughter house where they were killed at 1.00 p.m.[5] The technique was varied for the first two cows. The udder of one of these cows was minced and the minced material subjected to a pressure of 2000 pounds in an hydraulic press. This technique proved unfortunate in view of the fact that the udder material holds the milk secreted very tightly, it being almost impossible to press out any of the milk although it may be milked or drained out. In consequence the minced material was simply driven into the cloth and had to be extracted with water in the same manner as that used for the later seven cows. The second cow's udder was cut into small strips and allowed to drain and then these strips were put into the ton press. Here again it was impossible to press out any amount of secretion. The material was then extracted with water in the manner described for the other seven cows. The results of this unfortunate technique showed clearly that the musculature of the udder is such that milk may be extracted from it far more easily by the ordinary methods of milking with the cooperation of the cow than is possible with relatively large pressures applied under external conditions. The technique for the other seven cows consisted of milking these cows at 1.00 p.m. and 10.00 p.m. for 5 consecutive days, determining the milk flow and lactose percentage in the milk for each of these milkings. The cows were then killed at exactly the hour of previous milking, the last three being killed after milking, the other four being killed with the udder full of milk. The udders were then carefully dissected off, cut in strips, and drained for the milk which would quickly flow out. The remaining material was then ground and extracted with water three different times, about

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[4] That this opinion lacks foundation in fact in at least five cows is shown by the work of Swett, W. W., Relation of conformation and anatomy of the dairy cow to her milk and butterfat-producing capacity. Udder capacity and milk secretion, *J. Dairy Sc.*, 1927, x, 1.

[5] It is a pleasure to acknowledge our indebtedness to Penley's Packing Company, Auburn, Maine, for their cordial cooperation.

50 pounds of water being used in each extraction, the fluid material being drained through cheese-cloth bags. The remaining extracted udder material was then dried, ground, and the little sugar which remained in it was determined by first removing the fat with gasoline and then extracting with water. Printing cost allows only publication of the totals.

Table I shows the milk production of the cows for the 1 o'clock milking and the 10 o'clock milking for the 3 days previous to their slaughtering.

TABLE I.

Average Milk Production in Pounds for the 1 p.m. and 10 p.m. Milking, for the 3 Days Previous to Slaughter. August, 1926.

Cow No.	Average milk yield	
	1 p. m.	10 p. m.
111	12.9	7.4
124	17.9	10.0
132	15.6	10.3
136	15.7	13.8
148	13.5	8.3
154	11.7	5.9

TABLE II.

Average Milk Production in Pounds for the 1 p.m. and 10 p.m. Milking of Cows Slaughtered Just Following 1 p.m. Milking. August, 1926.

Cow No.	Average milk yield		
	1 p. m.	10 p. m.	Last 1 p. m. milking
97	4.7	2.2	2.8
114	4.1	2.8	3.6
153	10.8	6.9	9.6

Table II shows the milk production of the cows, at the 1 o'clock and 10 o'clock periods, which were slaughtered just following milking.

The milk productions in Table II are lower than those in Table I. The cows had to be shipped 10 miles to the slaughter house before they were killed, so that this disturbance probably played some part in their milk production before slaughter, tending to decreased secre-

tion and to retention of that which was secreted. This is noted in the fact that the milk production of Cow 97 was 1.9 pounds less than her 1 o'clock average for the 3 previous days. No. 114 had 0.5 of a pound less than the average of the 3 previous days, and No. 153, 1.2 pounds less than the average of her earlier milkings. It was found that 0.8 of a pound of milk could be drained out of the udder of No.

TABLE III.

Average Lactose Per Cent for 1 p.m. and 10 p.m. Milking for 3 Days Previous to Slaughter. August, 1926.

Cow No.	Average lactose	
	1 p. m.	10 p. m.
	per cent	*per cent*
111	4.62	4.60
124	4.78	4.72
132	5.28	5.33
136	5.05	5.10
148	4.81	4.89
154	4.84	4.90

TABLE IV.

Average Lactose Per Cent for 1 p.m. and 10 p.m. Milkings of Cows Slaughtered Just Following 1 p.m. Milking. August, 1926.

Cow No.	Average lactose		Lactose
	1 p. m.	10 p. m.	Last 1 p. m.
	per cent	*per cent*	*per cent*
97	3.98	4.03	3.80
114	4.72	4.80	4.85
153	4.50	4.58	4.50

97, although the milking had been performed very carefully. Cows 114 and 153 showed some milk left in the udder, but not enough to weigh. This milk was allowed to go into the determination of the sugar content in the udder, following milking.

Tables III and IV show the percentages of lactose found in the daily milkings of the cows given in Tables I and II. It will be noticed that there appears to have been little or no change in the lactose per-

centages of Cows 97, 114, and 153 from the average for the milk of the 3 previous days. It may be concluded from this that any excitement incident to the trip to the slaughter house for these cows had no effect in changing the lactose content of their milk.

The total lactose for all of the extractions is shown in Table V. This total lactose divided by the average lactose percentage for the 1 o'clock milk yield of the 3 preceding days gives the amount of milk necessary to account for this total amount of lactose. Throughout, the materials added checked well with those extracted. The udder material remaining after extraction shows an average of less than .04 per cent lactose.

TABLE V.

Weight of Lactose Extracted from Udders and Milk Equivalent of Lactose, Pounds. August, 1926.

Cow No.	Weight of total lactose	Milk equivalent
111	.718	15.5
124	.931	19.5
132	.774	14.6
136	.798	15.6
148	.614	12.8
154	.538	11.2
97	.247	6.2
114	.140	3.0
153	.150	3.3

The lactose extracted from the udder, in the case of the unmilked cows, represents the lactose contained in the milk which would be drawn on milking and the lactose which would remain in the udder. For the cows milked just before death the lactose represents the milk which could not be drawn from the udder by milking. This, converted into pounds of milk, represents for Cow 97, 6.2 pounds; for Cow 114, 3.0 pounds; for Cow 153, 3.3 pounds. The large amount of milk remaining in the udder of Cow 97 calls for comment. After the milking was completed and the udder dissected off about 3/4 of a pound of milk was found in this udder which could easily be drained out of it. This milk was held up by the cow even though the milking was performed carefully by an experienced milker. It will be noticed

that no such amount of milk was found in the udders of the other two cows, 114 and 153. In fact, not over .1 of a pound of easily drained milk was left in these udders. Should the 3/4 of a pound of free milk be left out of consideration there is still 5.4 pounds found in this udder. The total milk of Cow 97 is consequently much larger than her previous milk yield would lead one to expect. How this cow manufactured this extra 2 or 3 pounds of milk the authors do not know. These three cows' milk yields before killing, in comparison with the average of the 3 previous days, were 1.9 pounds less than would be expected for Cow 97, .5 of a pound less for Cow 114, and 1.2 pounds less for Cow 153. The reduction in milk yield seems to be accounted for by the strange conditions under which the milking took place and the nervous excitement previous to milking. If these values be subtracted from the milk found in the udder, determined as lactose, Cow 97 has 4.3 pounds of milk remaining in the udder; Cow 114, 2.5 pounds; and Cow 153, 2.1 pounds. Cows 114 and 153 appear to check nicely. Cow 97 has about 2 pounds more milk in the udder than would be expected on the basis of the other results. Whether the average of the three cows should be used or only the average of the last two is perhaps a question. If the average of the three cows is used it is found that the udder contains 3 pounds of milk when it is supposedly milked dry. The milk found in the udders of the six remaining cows determined as the lactose equivalent, represents the milk which would be drawn in normal milking plus that which was retained in the udder after the cow was considered dry. If these figures be compared with the amount of milk which the cows gave it will be noted that they correspond fairly well. Thus the average milk production of Cow 111 was 12.9 pounds while the amount accounted for is 15.5 pounds, that for Cow 124 was 17.9 pounds while the milk accounted for is 19.5 pounds, that for Cow 132 was 15.6 pounds while the milk accounted for is 14.6 pounds, for Cow 136 the milk production was 15.7 pounds while the milk accounted for is 15.6 pounds, for Cow 148 the milk production was 13.5 pounds while that accounted for was 12.8 pounds, and finally the milk production of Cow 154 was 11.7 pounds while that accounted for was 11.2 pounds. It will be noted throughout that the amount of milk accounted for and the amount of milk drawn from the udder correspond fairly

closely. They show, furthermore, the following relative relationships: the higher milking cows show the larger amount of milk in their udders; the lower milking cows, the lower amount of milk in their udders; with the medium yielding cows between the two extremes. The average milk production for the six cows was 14.6 pounds; the average amount of milk accounted for for these cows was 14.9 pounds. It is to be remembered that of this milk accounted for on the basis of lactose there is probably remaining in the udder after milking between 2 and 3 pounds of milk. Thus from the 14.9 pounds accounted for on the basis of lactose there should be subtracted between 2 and 3 pounds due to the amount of milk which it is impossible to milk from the udder. The comparison of the amount of milk accounted for should be therefore between 12 and 13 pounds as contrasted with the 14.6 obtained. All these results show clearly that at actual time of milking between 80 and 85 per cent of the milk can be accounted for in the udder of cows milking up to 30 pounds of milk a day.[6] The experiment therefore points to the conclusion that 20 per cent is a maximum and 10 to 15 per cent, a probable value for the amount of milk which may possibly be secreted in the udder during the time of milking.

This value is considerably less than that obtained by Maxwell and Rothera in their experimental work. Their results are largely dependent upon the accuracy with which the lactose in the milk of the cat is represented by their assumed value of 5.07 per cent. Gaines and Sanmann cite Folin, Denis, and Minot's analyses on 19 samples

[6] The technique of the experiment is such that it is necessary to make the sugar analysis over a period up to 4 days from the time of extracting the udder. The extracted materials were carefully preserved on ice and 10 drops of formalin added to each pint of extract. This method of preservation has shown little change in the sugar content although that possibility is to be considered. The acidity test was throughout all of the experimental work between .005 and .19 per cent. The milk acidity from the cows was throughout between .1 and .13 per cent. There is then a chance that some of the sugar in the material extracted from the udder was converted into acid before it could be read. Another and perhaps more serious chance to decrease the lactose accounted for in comparison with the milk of the 3 previous days is a reduced milk secretion on the day of killing incident to the trip to the slaughter house. Thus all the factors tend to reduce the milk accounted for in contrast to the average of the 3 previous milkings.

of the milk of three cats. These data give a range in percentage of lactose from 2.3 to 4.0. For 26 samples on four cats the average was 3.4. Should 3.4 be used in place of 5.07 as the lactose per cent of cats' milk, the total secretion of the mother cat as extracted by the kittens is accounted for.

Our data are in general accord with those of Gaines and Sanmann. Their technique on Cow 2 is, we believe, better than ours in that there is less chance of losing lactose in the manipulation of the udder and we are inclined to the view that our results show somewhat less lactose than was actually present in the udders.

The available information thus indicates that the mammary gland at time of milking contains the majority of the lactose to be secreted in milk.

Relation between the Size of the Mammary Gland and the Milk It Secretes.

The relation which exists between the size of a gland and the size of the product which it manufactures is an almost unstudied problem of gland physiology. In fact the problem has been approached only by judges of dairy cattle. These qualitative data have led to the assertion that the size and quality of the udder does to some extent indicate the productive capacity of the cow. The records of these nine cows in the experiments herein described furnish unique, fairly exact evidence on this problem. The correlation which can be derived from these data is admittedly open to a very large probable error. It does, however, furnish an important guide to the results which might be expected on more extensive data. The material also has the advantage that the determinations are quantitative and relatively accurate as contrasted with those heretofore used. Statistically considered the results as treated are significant since $r = .96$, $t = 9.0$ and P for $N = 9 < .01$.

The most interesting comparison is that between the weight of the udder with the contained milk and the milk production which the cow normally gave. This measurement is also the most exact so far as the collection of the data is concerned. Table VI shows the relation of the milk yield to the weight of the udder and contained milk.

Table VI and Fig. 1 show that the average weight of the udder and

its contained milk increases as the milk production which the cow is then giving increases. This increase amounts to about 1.2 pounds

TABLE VI.

Total Weight of Udder and Contained Milk Contrasted with the Cow's Average Milk Yield at the Same Period.

Cow No.	Average milk yield	Weight of udder and contained milk
114	4.1	16.6
97	4.7	18.8
153	10.8	26.1
154	11.7	24.2
111	12.9	29.3
148	13.5	27.2
132	15.6	28.7
136	15.7	30.6
124	17.9	36.0

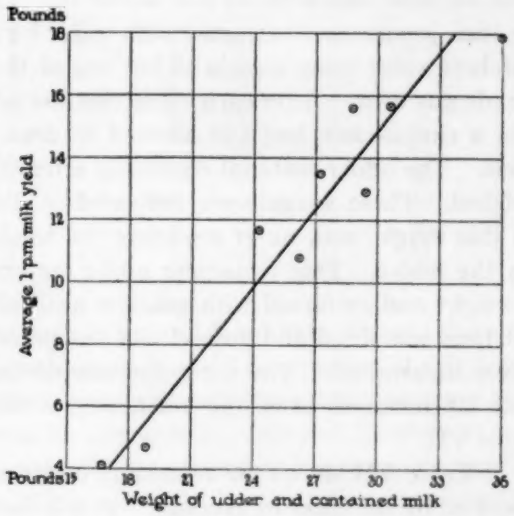

FIG. 1. Relation between average 1 p.m. milk yield and the weight of the udder and contained milk.

for the weight of the udder and its contained milk as the cow increases in milk production 1 pound, the increase being fairly regular over the

entire range. From this fact it may be concluded, tentatively, as the probable errors are large, that for creating a pound of milk between 10.00 p.m. and 1.00 p.m. (15 hours), .2 of a pound of secreting mammary tissue would be necessary. On the basis of these few observations the relationship between milk yield and the mammary gland size appears to be linear. In view of this fact, if the line of general relationship is extended to the point of no milk production, the udder would weigh about 12 pounds. On the basis of the results for Cows 97, 114, and 153 such an udder would still contain 2 to 3 pounds of milk. The mass of udder tissue in the practically dry cow would consequently be 9 to 10 pounds. This might be conceived of as connective tissue supporting the glandular structure, furnishing the surrounding tissue for the teats and alveola spaces.

The Remaining Udder Material.

As indicated earlier the udder with its contained milk was first cut and drained of the milk which would flow out of it. The remaining material was then ground and extracted with water for three extractions, 50 pounds of water being used in all but one of the extractions where 40 pounds was used. After each extraction the udder material was placed in a cheese-cloth bag and allowed to drain for varying lengths of time. The udder material remaining after this treatment was then weighed. These weights are indicated in Table VII. A large part of this weight was water replacing the soluble materials washed from the udder. This remaining udder material was dried to constant weight and extracted with gasoline until all the fat was removed. It then was dried and ground and the percentage of remaining lactose determined. The determination of the percentage of water and fat removed from the remaining udder material is shown in Table VII.

Column 2 of Table VII shows the remaining udder material after it has been washed by the three extractions. It will be noticed that the amount of this material has a fairly close correlation to the yield of milk which the cows are giving.

The percentage of water and fat which was found in this material is shown in the third column. This water and fat varies between 85 and 91 per cent of the udder material. As would be expected from

the fact that the length of time in draining the udder was not constant for each cow, this percentage variation is quite random.

The weight of the remaining udder substance after the extraction of water and fat is shown in the fourth column of the table. The amount of udder substance varies between 1.4 and 2.3 pounds. On the basis of the total weight of the udder and its contained milk this dried material, gasoline- and water-soluble-free, is 5 to 10 per cent of the udder weight.

If the weight of the remaining udder substance is compared with the yield of milk which the cow is able to give, but little correlation is found. This lack of correlation is quite likely caused by the fact

TABLE VII.

Remaining Udder Material after Extraction, and Percentage of Water and Fat Removed from the Same.

Cow No.	Remaining udder material	Percentage of water and fat	Weight of remaining substance	Milk yield total
111	15.7	87.7	1.93	20.3
124	16.9	89.6	1.76	27.9
132	14.5	87.3	1.84	25.9
136	16.5	86.1	2.29	29.5
148	15.5	90.8	1.43	21.8
154	15.2	89.3	1.63	17.7
97	12.0	84.7	1.84	6.8
114	11.0	87.4	1.39	6.9
153	14.5	89.6	1.51	17.8

that these cows were, in general, producers of about the same capacity. The lower productions of 97 and 114 represent the drying off of these cows. This insoluble udder substance may be regarded as largely supporting tissue for the secreting cells and therefore of a more or less permanent nature. By the method of treatment this material appears to be of about the same weight for the cows secreting milk and those which are approaching the dry period.

SUMMARY.

The results herein presented furnish exact critical evidence for the conclusion that the most of the milk is present as such in the udder of

dairy cattle at the time of milking. The amount of milk which may be secreted during milking cannot, on the basis of these results, be over 20 per cent of the milk yield of the cow.

The results show clearly that the size of the udder measures closely the amount of milk which the cow is able to secrete.

The results indicate that about 1/5 of a pound of secreting tissue is necessary for the secretion of a pound of milk during a period of 15 hours. The weight of the udder during the period that the cow is dry appears to be between 6 and 8 pounds.

THE EFFECT OF TEMPERATURE UPON SOME OF THE PROPERTIES OF CASEIN.

By VLADIMIR PERTZOFF.

(From the Department of Physical Chemistry, Laboratories of Physiology, Harvard Medical School, Boston.)

(Accepted for publication, May 25, 1927.)

I.

INTRODUCTORY.

The considerable changes which occur in the behavior of living matter when subjected to high or low temperatures raise interesting questions as to how the temperature affects the behavior of certain substances present in nature.

Casein, purified at the point of its maximum flocculation, is able to bind base, forming a salt soluble in water. The amount of casein which passes into solution is not only dependent upon the amount of base added, but also upon the temperature.

We propose to study the interdependence of three variables: the base added, the casein dissolved, and the temperature.

The properties of casein when dissolved in alkali and combined with base have been studied by a large number of investigators and by different methods. The results obtained may conveniently be considered in terms of the different equivalent combining weights for base that they have ascribed to casein.

II.

The Equivalent Weight of Casein.

The combining weight of casein was investigated before the development of the electrolytic dissociation theory. In 1865, Millon and Commaille (1, 2) stated: "...la caséine, matière unique, s'unirait sans doute à la plupart des acides minéraux et organiques, et si toutes ces combinaisons étaient réellement bien définies, il ressortirait de leur

examen une connaissance plus éxacte de la caséine, de sa formule, de son équivalent et de son affinité."

Millon and Commaille assigned to this protein the formula weight of C_{108} H_{97} Az_{14} O_{29} for its equivalent (1), which upon calculation yields 2050 gm. This number, as we shall see, is in excellent agreement with the equivalent weight of this protein obtained by modern

TABLE I.

The Titration of Casein with Base.

Investigator (1)	Year (2)	Method (3)	Equivalent of base $\times 10^{-4}$ bound by 1 gm. casein (4)	Gm. of casein bound by 1 equivalent of base (5)
Söldner (4)	1888	To phenolphthalein	8.16	1230
Courant (5)	1891	" "	9.5	1050
Timpe (6)	1893	" "	9.35	1070
Laqueur and Sackur (8)	1903	" "	8.80	1135
Matthaiopoulos (9)	1908	" "	8.85	1130
Hart (10)	1909	" "	9.25	1080
Pfyl and Turnau (11)	1914	" "	8.75	1145
Peroff (12)	1921	" "	8.20	1220
Bleyer and Seidl (13)	1922	" "	8.77	1144
Average				1124
Robertson (15)	1910	Electrometrically at pH 8.50	8.0	1250
Loeb (14)	1921	" " " 8.50	6.6	1520
Cohn (23)	1925	" " " 8.50	6.3	1590
Robertson (15)	1910	" " " 7.00	5.1	1960
Loeb (14)	1921	" " " 7.00	4.3	2340
Cohn (23)	1925	" " " 7.00	5.0	2000

workers. It seems plausible to give credit for the discovery of this important number in the chemistry of casein to these investigators, although this discovery was of a somewhat accidental nature.

Hammarsten (3), in 1877, greatly improved the method of preparation of casein, which with few modifications is still used by modern investigators. He (3) also discovered that casein decomposes $CaCO_3$ liberating CO_2, which indicated that this protein is an acid.

Titration of Casein to Indicators.—Many investigators have studied the acid properties of casein, titrating it with base to a suitable indicator. Phenolphthalein was chosen most often, as the appropriate method of titrating a weak acid, casein, and a strong base.

Söldner (4) found that 8.3 cc. of 0.10 N mono- or divalent base were required to bring 1 gm. of casein to neutrality to phenolphthalein. Since Söldner's research, Courant (5), Timpe (6), Bechamp (7), Laqueur and Sackur (8), Matthaiopoulos (9), Hart (10), Pfyl and Turnau (11), Peroff (12) and Bleyer and Seidl (13) have titrated casein to indicators. Most of the results of these investigations are recorded in Table I. On the whole, these data agree fairly well with each other, showing the existence of a definite chemical substance, endowed with a certain base-binding capacity. Loeb (14) showed that the bases combined with casein in equivalent proportions whatever the end-point to which this protein was titrated.

Electrometric Studies upon Casein.—From our present knowledge of the chemistry of casein, the amount of base bound to phenolphthalein is of no fundamental importance; it is merely a single point on the titration curve of casein. Casein can bind far more base in more alkaline solutions. In 1910, Robertson (15) found that the casein preparations that he studied bound, at saturation, as much as 180×10^{-5} equivalents of base per gm. of casein.

Later electrometric estimates of the maximum base-combining capacity of various casein preparations (15–18) showed the existence of at least two modifications of casein: one binding about 180×10^{-5} equivalents of base per gm. and having a steeper titration curve and the other termed "unmodified" casein, having a maximum base-combining capacity of 138×10^{-5} equivalents and a flatter titration curve. The former of these modifications appears when a casein preparation, during the course of its purification, is subjected to an alkaline[1] treatment (18).

Solubility Studies upon Casein.—Robertson (19) studied the amount of base necessary to hold casein in solution. He found that 1.14×10^{-4} equivalents of monovalent base is sufficient to hold 1 gm.

[1] For a review of the subject, as well as the shape of the titration curves of caseins, consult Cohn, E. J., The physical chemistry of the proteins, *Physiol. Rev.*, 1925, v, 349.

of casein in solution (15). In systems containing $Ca(OH)_2$, the amount of base bound by casein was found to be 1.19×10^{-4} equivalents (20).

L. L. Van Slyke and Bosworth (21), using a very similar method, found that 1 gm. of casein forms a soluble compound with 1.10–1.115

TABLE II.

The Equivalent Weight of Casein.

Investigator (1)	Year (2)	Method (3)	Equivalent of base $\times 10^{-4}$ bound by 1 gm. casein (4)	Gm. of casein bound by 1 equivalent of base (5)
Robertson (19)	1909	"Saturation" monovalent bases	1.14	8800
Van Slyke and Bosworth (21)	1913	" "	1.125	8890
" " " " "	1913	"Saturation" divalent bases	2.25	4440
Millon and Commaille (1, 2)	1865	Analysis	4.88 and more	2050 and less
Long (24)	1907	Solubility	4.50	2220
Yamakami (22)	1920	"	(2.4)	(4.150)
Cohn and Hendry (25)	1923	"	4.76	2100
Greenberg and Schmidt (16)	1924	Electrochemical equivalent	4.96	2010
Carpenter (28)	1926	Formol titration	4.65	2140
Average of last six				2104
Cohn and Berggren (18)	1925	Maximum base-binding ("unmodified")	13.8	725
Robertson (15)	1910	Maximum base-binding ("Nach Hammarsten")	18.0	550
Cohn and Berggren (18)	1925	" "	18.3	545

$\times 10^{-4}$ equivalents of monovalent bases. Dialyzing the solutions of casein with divalent bases, they found that the amounts of these bases necessary to hold casein in solution were just twice the amounts of monovalent bases. In Table II we are referring to the method used by Robertson and by Van Slyke and Bosworth as the "saturation" method.

Yamakami (22) adding NaOH to casein and, filtering off the undissolved casein, found that 2.25 to 2.50 $\times$ 10^{-4} equivalents of base dissolve 1 gm. of casein. This upon calculation yielded 4.150 gm. for the equivalent weight of casein, that is the weight of casein bound by 1 gm. equivalent of base.

Long (24), in 1907, found that 5 gm. of casein will dissolve completely in 22.5 cc. of 0.1 N alkali, which upon calculation yields 2220 gm. for the equivalent weight of casein, based on these solubility measurements.

The solubility of casein in NaOH solutions was extensively studied by E. J. Cohn and Hendry (25). From their measurements they found the equivalent weight of casein to be 2100 gm. These investigators came to the important conclusion that the only determining factor in the solubility of casein with NaOH is the amount of base added. The amount of casein may be varied without affecting the solubility.[2] This value of the equivalent weight of casein has been recently confirmed by various methods. Greenberg and Schmidt (27), from the study of the electrochemical equivalent of casein, calculated the equivalent weight as 2015 gm. Carpenter (28), using the formol titration method, concluded "that one formula weight of amino-group is linked with carboxyl per 2150 gm. of casein."

Certain relationships exist between the equivalent weights obtained by various investigators from their solubility measurements. The equivalent combining weight with monovalent bases determined by the so called saturation method, 8800 to 8890 is just twice the weight obtained by the same authors with divalent bases, 4440. Half of this value, 2220, yields the equivalent combining weight determined by solubility, by transport number, or by formol titration. Again, one-third of the equivalent weight of casein determined by these methods is equal to the maximum base-combining capacity of "unmodified" casein, 725, and one-fourth to that of casein modified by alkaline treatment, 545. The researches carried out by many investigators thus reveal the existence of certain definite compounds of casein that exist under specified conditions.

[2] Compare with Linderstrøm-Lang's investigation (26) upon the solubility of casein with acids.

III.

EXPERIMENTAL.

Casein Preparation.—The proteins used in this investigation were prepared according to the modified (25) method of Van Slyke and Baker (29). The caseins were prepared from fresh unpasteurized milk. They were precipitated by the addition of HCl, and redissolved with NaOH. From fear of modification, we have never exposed our caseins to a hydrogen ion concentration less then pH 7.00. The casein solutions were passed through a Sharples centrifuge, filtered through paper pulp filter, and reprecipitated with HCl. This procedure, resolution, and reprecipitation, was repeated once or twice. Finally, the casein precipitate was extensively washed with distilled water until chloride-free. The procedure for the preparation of casein has already been reported in detail (25).

The Measurement of Solubility.—The solubility measurements were carried out very much as described in a previous communication from this laboratory (25). Samples of uncombined casein precipitates were placed in 100 cc. volumetric Pyrex flasks and the required amount of very dilute NaOH was slowly added. Next, the flasks were filled to the mark with CO_2-free distilled water and placed in a shaking machine for equilibration. All the reagents were always used at the temperature at which the experiment was set.

Two shaking machines were used in the process of equilibration of the protein with NaOH. One, less efficient, was used in the experiments at 25° and at 37°C. The other, at least three times more efficient than the former, was used for all experiments at other temperatures. The times for equilibration given in the tables describing our experiments are therefore not strictly comparable to each other.

After the systems containing casein had reached equilibrium, they were filtered through No. 42 Whatman's paper filters. The filtration was carried out at the temperature of the experiment, except in those done at 49°. In this investigation, the filtration was carried out at about 20°C. As we shall see in a subsequent section of this paper, this difference of temperature did not interfere with the measurements.

Aliquot parts of the filtrates were then analyzed for nitrogen by the Kjeldahl method. Allowance was made in calculations for the small amounts of nitrogen present in the reagents. The analyses were carried out in triplicate. In all calculations we used the factor 6.40 for converting the amount of casein nitrogen into the amount of the protein,—that is, the per cent of nitrogen in casein was assumed to be 15.62.

If one calculates the results of the analyses on the basis of the solution, without taking into consideration the precipitate, the result will be a figure slightly higher than the true one, as the total volume of a suspension is made up of two parts: the volume of the solution and the volume of the precipitate.

In all our experiments the amount of casein in the form of a precipitate was less than 1 per cent, and since the density of casein precipitate is about 1.26 (30) the error is less than 1 per cent and can be neglected for the present.

IV.

The Effect of Temperature on the Solubility of Casein in Water.

The solubility of casein in water at the point of minimum solubility has been the subject of several investigations. Bechamp (7) found that freshly precipitated casein, free from acid, was soluble in water. According to this investigator, the solubility is dependent both upon time and temperature. From 0.24 to 1.005 gm. of casein was found to dissolve in 1 liter of water between the temperature range of 15° and 25°C. In order to obtain the maximum solubility, Bechamp found it necessary to triturate casein with water for 52 hours. Under these conditions, at 20°C., casein was found to be soluble in water to an extent of 1.005 gm. per liter of solution.

Osborne (31), Laqueur and Sackur (8), and Robertson (32) believed that no appreciable amount of casein dissolved in water. Yamakami (22) triturated casein with distilled water and after leaving the suspension standing for a considerable period of time at low temperature, found from 0.0118 to 0.0156 gm. of casein per 100 cc. of the filtrate. He, however, thought that the reason for this phenomenon was purely manipulative.

Cohn (33), after a prolonged agitation of suspension of casein in water, found that this protein dissolves to a constant and characteristic amount, the amount dissolved being independent of the amount of the casein in the suspension; 0.11 gm. of casein according to this investigator, is dissolved in 1 liter of water at 25.0°C.

As we see, the results of these investigations disagree widely. It is quite likely that the earlier measurements of the solubility of uncombined casein in water are incorrect. Accurate measurements became possible after the development of the modern knowledge of the isoelectric points of proteins.

It was of interest to investigate the effect of temperature upon the solubility of this protein in water as compared to the effect of this factor on the capacity of casein to bind base. If these two phenomena are unequally affected by the temperature, they are likely to be of different physicochemical nature. If, conversely, they are equally increased or decreased by the temperature, they probably are functions of a single physicochemical property of the protein.

The results of our investigation on the solubility of several preparations at 5.0°C. and of Casein Preparation XXVII at 25°C. are reported in Table III.

From the results of these measurements the solubility of uncombined casein in water at 5.0°C. may be taken as being equal to about 0.70 ± 0.10 mg. of N per 100 gm. of water, or, in terms of the protein, 0.045 gm. are dissolved per 1000 gm. of water.

Table III also includes an investigation of the solubility of Casein Preparation XXVII at 25°C. It appears from our experiments that this casein preparation dissolved to a slightly greater extent than the value given by Cohn (33) for the solubility of this protein in water. It seems advisable, therefore, to widen the limits of the solubility of casein in water, assigning to this solubility 2.00 ± 0.30 mg.

of nitrogen per 100 gm. of water at 25°C., or, in terms of casein, about 0.13 gm. of casein are soluble per 1000 gm. of water.

We also attempted to measure the solubility of casein in water at 37°C., but found that the solubility at this temperature, unlike that at 25°, was dependent upon time, indicating, presumably, a progressive hydrolysis of the dissolved casein.

From the data at hand, we may conclude that the solubility of casein in water is dependent on the temperature: an increase of 20°C. nearly triples the solubility of this protein in water.

A comparison of the increase of the solubility in water of casein with the corresponding change in the capacity of casein to bind base (Fig. 2) indicates that these phenomena quantitatively are not identical: the change in the acid properties

TABLE III.

The Solubility of Casein in Water at Various Temperatures.

Temperature °C. (1)	Casein preparation (2)	Experiment No. (3)	Time for equilibration (4)	Solubility: Mg. N dissolved per 100 gm. of H_2O (5)
			hrs.	
5.0±0.5	XXIV	26	45	0.80
	XXV	25	45	0.60
	XXVII	29	40	0.64
	"	34	20	(0.40)
25.0±0.1	"	32	70	2.35
	"	31	62	2.30
	"	41	23	2.20

of casein, as measured by its solubility in base, is less than the change in the solubility of this protein in water, which suggests that these phenomena may depend upon independent chemical reactions.

V.

The Effect of Temperature upon the Solubility of Casein in Base.

In 1888, Söldner (34) noted that the salts of casein with Ca, Ba, Sr, and Mg become opalescent when heated to about 40°C. The opalescense disappeared upon the cooling of the solution. Osborne (31) confirmed these observations and also found that Li caseinate shows a very slight turbidity when warmed; ammonium caseinate, however, showed no sign of precipitation. Osborne explained this phenomenon

in terms of hydrolysis of the protein salt: on heating the solution, casein is formed, which being insoluble, produces the opalescense. In 1908, in an extensive investigation upon the effect of temperature upon the solubility of cases with bases, Robertson (35) found that, unlike the systems containing casein and bivalent bases, those containing this protein and monovalent bases are affected by the temperature in the opposite manner: with the rise in temperature the solubility increases. Robertson correctly pointed out that the hypothesis

TABLE IV.

The Solubility of Casein in Alkali at Various Temperatures.

Robertson (35).

Amount of base added equivalent $\times 10^{-5}$	Solubility: Gm. of casein dissolved per 100 cc. at							
	21°	36°	46°	54°	60°	66°	81°	88°
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
KOH								
23.0	0.46					0.52		
46.0	0.92	0.92	1.04		1.34	1.27	1.34	1.13
69.0	1.38					2.11		
92.0	1.85			2.77		3.05		
LiOH								
44.0	0.89	0.86			1.28	1.14	1.60	1.20
88.0	1.77			2.62		2.95		
$Ca(OH)_2$								
45.0	0.90	0.72			0.65	0.63	0.64	0.63
90.0	1.80			1.35				

of Osborne did not explain the effect of temperature upon the solubility of casein in monovalent bases.

Robertson (35) concluded that:

"the facts are much more readily explained on the supposition that the effect of temperature consists in shifting the equilibrium,

$$HXOH + HXOH \rightleftharpoons HXXOH + H_2O$$

toward the right so that a given amount of alkali (since it is associated with a molecule of nearly double that weight) neutralizes nearly twice as much casein

at 66° as it does at room temperature (21°). The marked diminution in the solubility of casein in $Ca(OH)_2$ solutions, which occurs on raising the temperature, can be explained by supposing that the salt $Ca(XXOH)_2$ is insoluble, while the salt $Ca(XOH)_2$ is soluble."

However, Robertson's theory, essentially based on the supposition that the protein's ultimate particle could be easily broken into two, has met with considerable criticism. Its application to the ionization of the proteins was unquestionably disproved by Greenberg and Schmidt, who, in a series of experimental studies (27, 36) upon casein, have shown that the behavior of this protein can be explained upon the assumption of the formation of a single protein ion.

Furthermore, Robertson's own data (Table IV) do not favor his theory: an increase of temperature from 21° to 60° does not double the solubility of casein in base and the amount of protein dissolved at 60° can hardly be increased by a further rise of temperature. The phenomenon seems to be of a far more complex nature.

Although we disagree with some of the aspects of Robertson's interpretations of his experiment, the data themselves, as we shall see, are in excellent agreement with recent solubility measurements upon casein. Since in a further discussion of this subject we shall extensively use Robertson's data, we shall first reproduce his table (Table IV), replacing the "concentrations of base" by the "amount of base added," and secondly, give a short description of his experimental procedure.

A given amount of base (35), diluted to 100 cc. with distilled water, was warmed to the desired temperature in a thermostat, which was kept constant within 0.5°. Then three times the amount of casein which would be dissolved by the given amount of alkali at room temperature (*i.e.* 3 gm. to every 5 cc. N/10 alkali) was introduced and the mixture left in the thermostat for from 30 to 40 minutes, being vigorously shaken at frequent intervals. The resulting solution was then filtered at the same temperature and the filtrate allowed to cool.

The estimate of the amount of casein dissolved was made by titrating the solution to phenolphthalein. Knowing that 0.125 gm. of casein bind 1 cc. of 0.1 N alkali to this indicator, and the amount of alkali already added to the sample, Robertson was able to estimate the amount of casein dissolved.

TABLE V.

The Solubility of Casein in NaOH at Various Temperatures.

Temperature °C. (1)	Casein preparation (2)	Experiment No. (3)	Time for equilibration (4)	Mols NaOH × 10^{-6} added (5)	Solubility: Mg. casein N dissolved per 100 gm. H_2O (6)
			hrs.		
5.0±0.5	XXVII	42	42	0.50	1.05
	"	"	"	"	"
	"	33	18	1.00	2.75
	"	42	42	"	2.30
	XXV	19	4	2.00	5.1
	XXVII	33	18	"	5.50
	XXV	23	25	"	5.25
	XXVII	42	42	"	5.50
	XXV	19	4	4.00	8.87
	XXVII	33	18	"	9.03
	XXV	23	25	"	8.45
	XXV	19	4	6.00	12.8
	XXVII	33	18	"	12.9
	XXV	23	25	"	13.1
37.0±0.5	XXVII	35	45	2.00	8.95
		36	50	"	9.60
	"	35	45	4.00	16.2
	"	36	50	"	15.7
		38	100	"	(17.9)
	"	35	45	6.00	22.7
		36	50	"	22.4
49.0±0.3	"	43	2	10.00	29.8
		"	4	"	29.5
	"	"	2	20.00	71.8
	"	"	"	30.00	111
	"	"	4	"	114

The casein solutions subjected to such high temperature as 88°C., used in certain of Robertson's experiments, are indeed likely to undergo a hydrolytic scission. Robertson (35) found that 46×10^{-5} equivalents of KOH at 88° after half an hour dissolved 1.13 gm. of casein. The same solution kept for 3 hours dissolved 1.25 gm. The error, according to Robertson, would be about 0.04 gm. in half an hour at 88°.

At lower temperatures, the error must be still smaller, and at 49° as our measurements show (Table V), increasing the time for equilibration from 2 to 4 hours hardly changed the solubility of casein in NaOH.

It is therefore quite probable that up to 80°C. the hydrolysis of casein does not appreciably affect the solubility measurements, provided of course the solutions are not exposed to high temperature for a long period of time.

The results of Robertson's investigation at 21° and at 66° are reproduced in Fig. 1. For comparison, on the same chart are plotted the results obtained by Cohn and Hendry (25) with NaOH at 25°. It is evident from this graph that the results of Robertson's measurements yield an excellent straight line. The equivalent weight of casein, calculated from these experiments, is 2000 gm. at 21°C. Furthermore, KOH, LiOH, and Ca(OH) dissolve casein at this temperature in equivalent proportions.[3]

The equivalent weight calculated by us from Robertson's experiments is in close agreement with the one obtained by Cohn and Hendry. The former is 2000; the later 2100. The agreement is within about 5 per cent. The comparison is possible because (Table IV, Fig. 4) the solubility of casein with monovalent bases in this range is independent of the temperature, the combining weight remaining constant throughout the range from about 21° to 37°.

[3] Robertson formerly held the same opinion. Later, however, he gave it up in favor of the evidence brought about by the experiments of Van Slyke and Bosworth (21). The statement that casein is dissolved by $Ca(OH)_2$ in equivalent proportions is not strictly correct. As our unpublished measurements indicate the relationship obtained by Robertson holds true only under certain experimental conditions. Unlike the solubility with monovalent base the solubility of casein with divalent base is a function of the amount of casein in the system. Such systems will be considered in a separate communication.

It is of interest to consider these two sets of experiments from the point of view of the equilibrium condition. The time of equilibration in Robertson's experiments was about 35 minutes. The corresponding

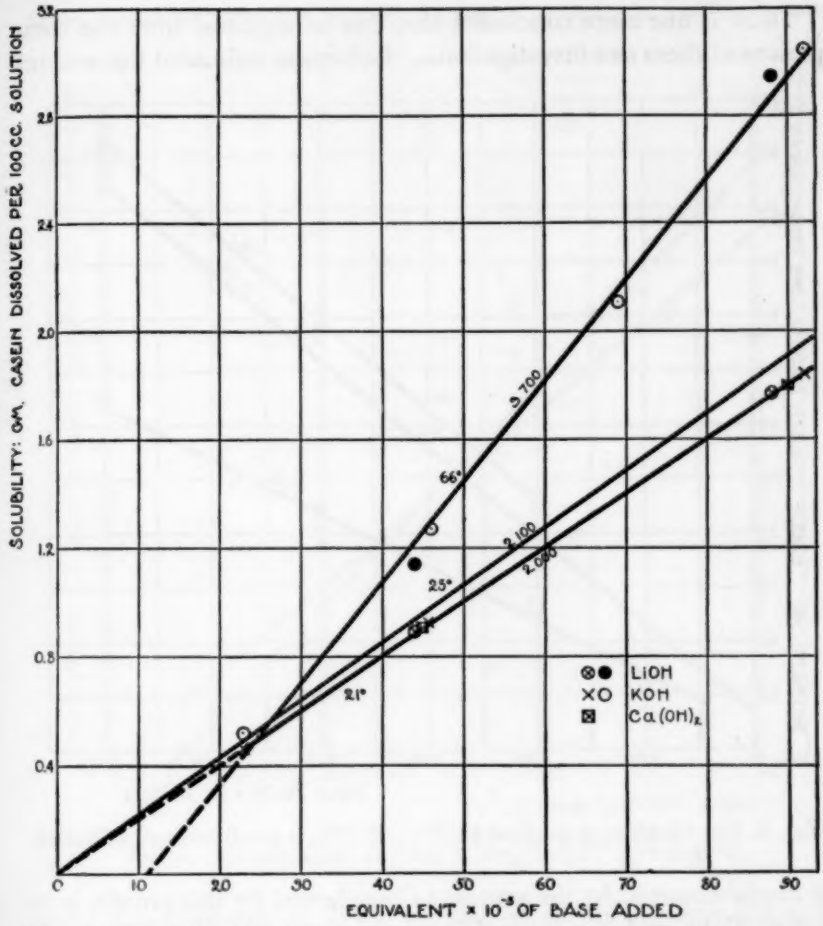

FIG. 1. The solubility of casein in bases at 21° and 60°C. according to Robertson (35) and at 25°C. according to Cohn and Hendry (25).

time in Cohn and Hendry's experiments varied from 24 to 72 hours (25). It is evident that a 150 fold variation of the time of equilibration had practically no effect upon the solubility of the casein. We may

conclude from this that, first, casein is readily soluble in alkali; and, second, that soon a stable state is reached, the solubility of the casein being practically independent of time. The latter is a criterion for the equilibrium conditions.

There is one more conclusion that can be obtained from the comparison of these two investigations. Robertson estimated the amount

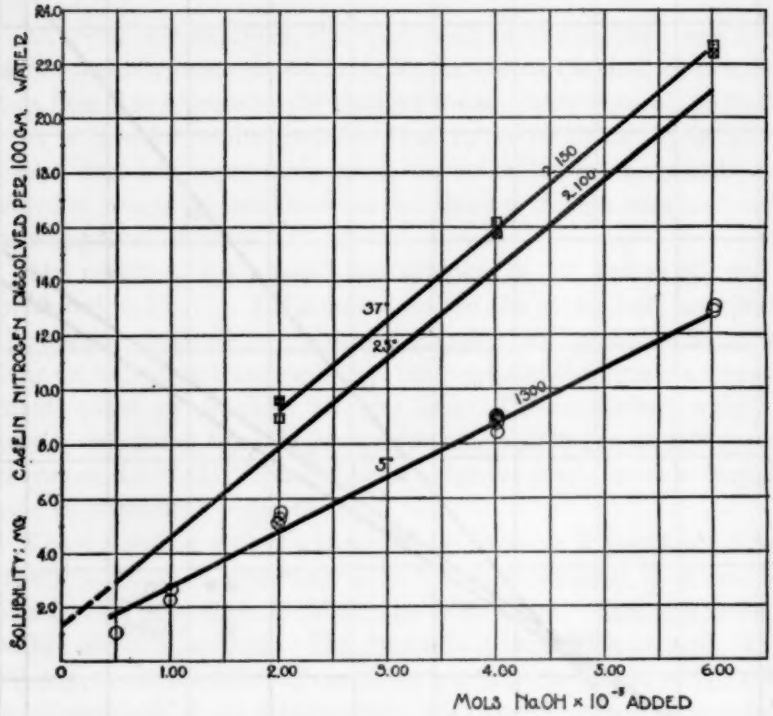

FIG. 2. The solubility of casein at 5°, 25°, and 37°C. in small amounts of NaOH.

of casein dissolved by the amount of base bound by this protein in solution; Cohn and Hendry estimated the same quantity directly, by estimating the amount of casein in the liquid phase. The equivalent weights correspond graphically to the slopes of the lines relating the amount of base added, and the amount of casein in solution. The slopes of these lines in these two cases agree within 5 per cent, which evidently means that casein dissolves and binds base at the same rate.

To extend and supplement the observation of Robertson, we have carried out measurements of the solubility of casein in NaOH at various temperatures. These results are reported in Table V. They are graphically represented in Figs. 2 and 3, together with the calculated equivalent weights in gm. of casein. Cohn and Hendry's solu-

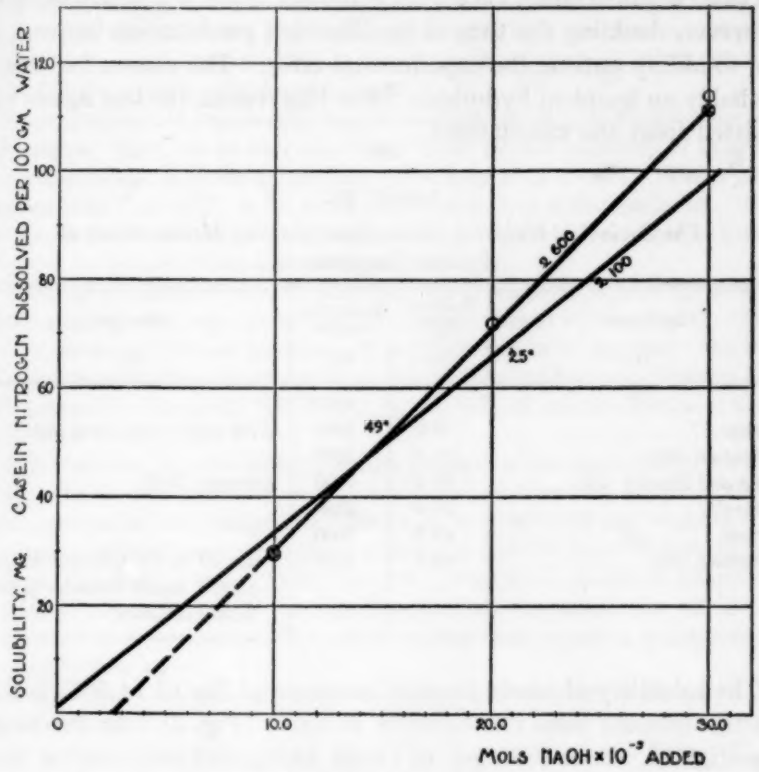

FIG. 3. The solubility of casein in NaOH at 25° and at 49°C.

bility measurements at 25°C. are also given on the same chart, for comparison.

For most of the experiments, Casein Preparation XXVII was used. This protein dissolved at 25.0°C. to the extent of 2100± 50 gm. per 1 mol of NaOH.

The time of equilibration for our individual experiments was re-

corded. At 5.0°C. we greatly varied the time of equilibration, since at low temperature we suspected a slower rate of reaction. As it appears from Table V, about 4 hours of continuous shaking is sufficient to bring this system to rest. The variation between individual measurements is within the experimental error. At 37°C. it appears that up to 50 hours of equilibration the solubility is not a function of time. However, doubling the time of equilibration produces an increase in the solubility outside the experimental error. The reason for this is probably an incipient hydrolysis. For this reason, the last figure was omitted from the calculations.

TABLE VI.

The Equivalent Weight of Casein from Solubility Measurements at Various Temperatures.

Investigator (1)	Temperature °C. (2)	Equivalent weight (3)	Remarks (4)
		gm.	
Author	5.0	1300	With small amounts of base
Robertson (35)	21.0	2000	
Cohn and Hendry (25)	25.0	2100	Average: 2070
Author	37.0	2150	
Author	49.0	2600	
Robertson (35)	66.0	3700	From 60° to 85° the equivalent weight again remains practically constant

The solubility of casein in small amounts of NaOH at 5°C. is distinctly different from its solubility at 25°C. (Fig. 2). In the range investigated, about 1300 gm. of casein are carried into solution by 1 mol of NaOH while according to Cohn and Hendry (25), 2100 gm. pass into solution per 1 mol of NaOH added, in this range at 25°C. The solubility of casein in small amounts of NaOH at 5°C. is unaffected by the amount of the saturating body. We have varied the amounts of this protein without appreciably changing the solubility. When casein begins to dissolve at 5°C., the slope of the solubility line may be, therefore, taken as being equally characteristic of this protein, as is the solubility of casein at 25°C.

In a separate communication we shall take up further consideration of this case in relation to the solubility of this protein in $Ca(OH)_2$.

In Table VI, we have compiled the value of the equivalent weights with monovalent bases at various temperatures.

Outside these data, we have the single determinations of Robertson (Table IV) from which there is no way to estimate directly the equivalent weight, since the origin of the solubility lines (Figs. 1, 2, and 3) varies with temperature and probably with the method used. These points cover the range from 21° to 60°C., as well as the temperatures higher than 66°C.

From these data the equivalent weights were calculated upon the following considerations. First, as we may note from Table IV, the solubility of casein in monovalent bases is probably constant in the range of 21° to 36°C. The origin of the line (Fig. 1) at 21° C. as far as we can estimate it, is at the zero point. Since no change in the solubility occurred, up to 36°C., it is plausible to assume that the origin of the line at this temperature has not changed.

A similar consideration holds true for the range of temperature from 60° to 80°C. The scattering of the experimental points is greater than at 21° (see Fig. 1). They do not show any decided tendency either to increase or to decrease. We have assumed therefore that the origins of the lines of the solubility measurements have not appreciably changed. The line at 66° C. (Fig. 1) cuts the abcissa at about 11.0×10^{-5} mols of base.[4]

It is therefore probable that the change of origin of the lines relating the solubility with the base added takes place at the temperatures from about 37° to 60°. The equation for this change as a function of temperature is not available. We have assumed it to be a straight line, and from this line calculated the intermediary corrections listed in column (3) of Table VII. Even if this relation in Robertson's experiments is not a straight line, the diversion from the straight line will only be a fraction of the correction while the correction itself is only a fraction of the amount of base added.

It is evident that the exact shape of the line is of no great importance to the calculated result.

Using these corrections for the origins, we have calculated in Table VII the equivalent weights for the single observations of Robertson.

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[4] Throughout this investigation we often refer to the origin of the solubility lines. This reference is made purely for the evaluation of the data. No physical significance should be attached to the values obtained by extrapolation, since it is always possible that with small amounts of base at high temperature casein would display a different combining weight, which might bring the origin nearer to the zero point. We have tried to measure the solubility of casein in small amounts of base at high temperature but found that these solutions underwent a rapid hydrolysis.

They are reproduced in Fig. 4, with the data compiled in Table VI. As we see, they agree satisfactorily with our own determination at 49°C.

If, however, this method of procedure seems not plausible enough, the conclusions obtained in the next section of this report can be deduced without the calculations of Table VII.

These calculations, as we believe, complete the quantitative formulation of the effect of temperature upon the properties of casein, and in this sense have their place.

From the physicochemical standpoint, it is of considerable interest to learn whether changes brought about by the temperature in the capacity of casein to bind base are reversible.

TABLE VII.

Calculation of the Equivalent Weight of Casein from Robertson's Experiments.

Temperature °C.	Mols × 10^{-4} of base added		Correction: Mols × 10^{-4} of base to be subtracted	Amount of LiOH or KOH added (corrected) Mols × 10^{-4} (2) − (3)	Casein dissolved	Equivalent weight (5) ÷ (4)
	KOH	LiOH				
(1)	(2)		(3)	(4)	(5)	(6)
					gm.	gm.
36	46.0		None	46.0	0.92	2000
36		44.0	None	44.0	0.86	1950
46	46.0		4.6	41.4	1.04	2500
54	92.0		8.2	83.8	2.77	3300
54		88.0	8.2	79.8	2.68	3350
60	46.0		11.0	35.0	1.34	3800
60		44.0	11.0	33.0	1.28	(3900)

While Ca caseinates become opalescent on heating, and the opalescence disappears on cooling, no such evidence is visible in the case of salts of potassium or sodium. In order to test the reversibility of this system, we brought some of the suspensions used in the experiment at 49°C. to a temperature of 5°C., at which temperature they were continuously shaken for about 200 hours. Then the suspensions were filtered and the amount of casein in solution determined as before.

No evidence of any decrease in the solubility of casein was noted. It is difficult to conclude from this that the reaction is irreversible, because it is always possible that the period of shaking of the suspensions was not sufficient.

From an entirely different source we have evidence that no permanent change occurs in casein, when this protein is subjected to a moderately high temperature. During the course of the preparation of casein, as a rule, the addition of acid or base was made at a tempera-

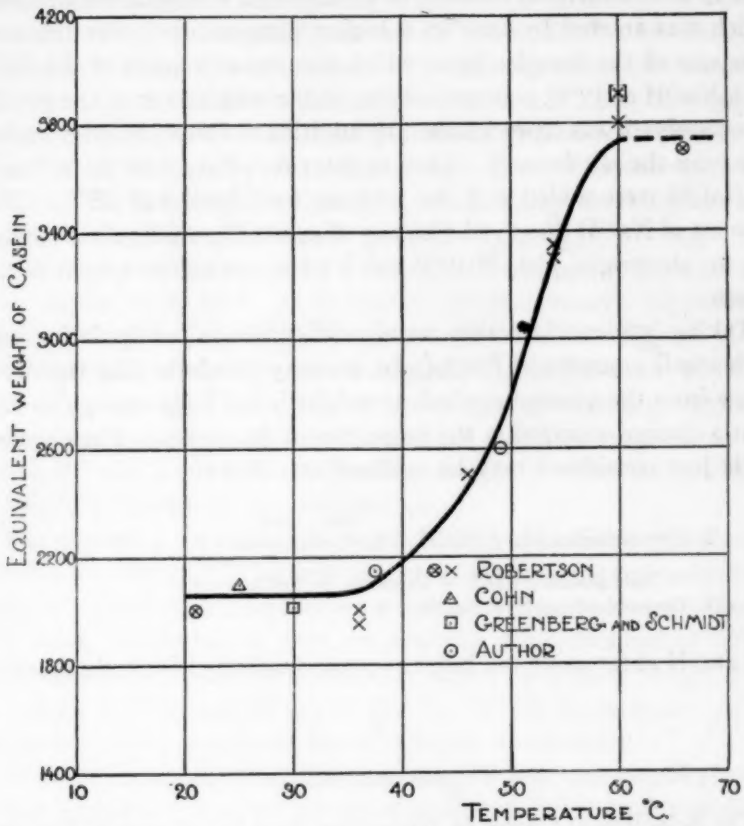

FIG. 4. The effect of temperature upon the equivalent combining weight of casein.

ture of 5°C; while the centrifugation and filtration was in most cases (including Casein XXVII) carried out at room temperature (about 20°C.). If the increase in the equivalent weights up to 2000 at about 20°C. is a change of an irreversible nature, evidently we would not be able to obtain a casein displaying a lesser equivalent weight at a lower

temperature. This is contrary to fact: the solubility of casein at 5° was found to be as low as 1300 gm. in some parts of the solubility curve. Therefore we must conclude that no permanent change occurs in casein, at least within the range of temperature from 5° to 20°C.

It is of considerable interest to extend such a conclusion to casein which was treated by base at a higher temperature. For this purpose one of the samples upon which the measurements of solubility with NaOH at 49°C. was carried out, and in which most of the protein was dissolved, was reprecipitated by addition of dilute HCl and washed free from the salt formed. Then to this precipitate 6.00×10^{-5} mols of NaOH were added and the mixture equilibrated at 25°C. This amount of NaOH dissolved 19.6 mg. of casein N, which, after correcting for the origin, yielded 1950 gm. for the equivalent weight of this casein.

Taking into consideration certain difficulties in manipulation with such small amounts of precipitate, we may conclude that the divergence from the average equivalent weight is not large enough to infer that a change occurred in the properties of the casein. The chemical cycle just considered may be outlined as follows:

$$\text{I. Uncombined casein} + \text{NaOH} = S_1 \xrightarrow{\text{heat}} S_2 \xrightarrow{\text{cool}} S_2 \rightarrow$$

$$\rightarrow \text{NaH caseinate} + \text{HCl} = H_2 \text{ casein} + \text{NaCl} \xrightarrow{-\text{NaCl}} \rightarrow \text{II. Uncombined casein} + \text{NaOH} = S_1$$

I and II as far as we can judge are quantitatively identical equations.

V.

DISCUSSION.

The Solubility of Casein in Monovalent Base in a Range of Temperature from 21° to 85°C.—Upon inspection of Tables IV, VI, and Fig. 5, the following seems to be evident. First, the base-binding capacity of a casein system, as measured by the solubility, is not a continuous function of temperature. The equivalent weight remains constant from 21° to 37°C., having the best representative value, in round figures, of 2100 gm. From 37°C. to about 60°C. the solubility again becomes a function of temperature, and the equivalent weight rises

from 2100 gm. to about 3700 gm. At a temperature higher than 60°C., casein with a rising temperature does not display any decided tendency either to increase its capacity to bind base, or to decrease it (Table IV). The scattering of the experimental points in the region of high temperature is greater than in the region of 21° to 37°C. A careful observation of the solubility of casein with KOH and $Ca(OH)_2$ (Table IV) favors this conclusion. The corresponding solubilities at 60°, 66°, and 81°C. from Table IV are 1.34, 1.27, and 1.34 gm. of casein. Upon inspection of Fig. 1, it is probable that the experimental point at 66° is slightly off of the average line. The corresponding point on the line is higher than 1.27, which brings these figures to a still better agreement in regard to their constancy.

A further justification of the existence of a plateau at the temperature higher than 60°C. is furnished by Robertson's experiments (Table IV) with $Ca(OH)_2$. It appears that the solubility of casein with $Ca(OH)_2$ is steadily decreased until the temperature of 60° is reached, after which the solubility remains remarkably constant.

It seems therefore plausible to conclude that at a temperature between 60° and about 85°C. there exists a region in which the solubility of casein is unaffected by temperature.

The numerical expression of the equivalent of casein at this plateau is probably 3700 gm. It is true that some of the single determinations show a slightly higher equivalent weight, but it must be remembered that from the statistical point of view the value obtained at 66°C., being based on several measurements, has a far greater weight than the single determinations at 60° and 81°, which furthermore, as we remember, were calculated by introducing a correction.

It seems reasonable, therefore, to conclude that the second plateau occurs when the equivalent weight of casein reaches the value of about 3700 gm.

In addition to the equivalent weight of casein determined by its solubility in monovalent bases, we can calculate, approximately, the amount of casein dissolved by one equivalent of $Ca(OH)_2$ at the temperature from 60° to 88°C. (Table IV). 1400 gm. of casein passes into solution per one equivalent of $Ca(OH)_2$ in this temperature range.

In a following communication we shall report an investigation upon the acid properties of paracasein. This modification of casein, accord-

ing to our experiments, has the equivalent weight with NaOH of 1450 gm. at 23° ± 2°C.

The combining weights 3700, 2100, 1450, and 1400 have one characteristic in common. They are all estimates of the solubility of casein in bases at temperature levels at which the base-binding capacity of casein seems to be unaffected by changes of the temperature, within certain limits.

The discontinuous behavior of casein toward the temperature suggests that these levels may be associated with definite changes in the casein molecule.

In Table VIII is found an attempt to identify such a change. It

TABLE VIII.

An Application of the Law of Multiple Proportions to the Equivalent Weights of Casein at Various Temperatures.

Temperature range °C. (1)	Base used (2)	Equivalent weight (3)	Equivalent weight divided by 720 (3)/720 (4)	Whole numbers ascribed to (4) (5)
		gm.		
60–85	Monovalent	3700	5.1	5.0
21–37	"	2100	2.9	3.0
60–88	$Ca(OH)_2$	1400	1.95	2.0
21–25	Paracasein-NaOH	1450	2.0	2.0

appears that these combining weights are common multiples of 720, common multiples within a few per cent. The common factor, 720, in its turn (Table II) is very nearly equal to the maximum base-binding capacity of an "unmodified" casein, which is 725 gm. at 20° (18).

The equivalent weight is a reciprocal measure of the acid or basic properties of a protein, since the greater the number of basic or acid groups involved, the smaller will be the weight of a protein combined with a given amount of acid or base.

It is therefore of interest for the estimate of acid properties of casein, to convert our combining weight into the amounts of base bound by 1 gm. of the protein. This has been done in column (4) of

Table IX. In column (5) of the same table we have calculated the ratio of the amount of base bound by 1 gm. of casein at saturation to the reciprocals of the remaining combining weights. The calculation yields a multiple relation, as we should certainly expect from the calculations of Table VIII.

It appears that 1 gm. of casein is completely dissolved at the temperature from 60° to 85°C. when it binds one-fifth of the amount of base necessary for its saturation at 20°C. At the temperature level from 21° to 37°C. the same amount requires one-third of the amount necessary for the saturation. Paracasein passes into solution when

TABLE IX.

The Law of Multiple Proportion and the Properties of Casein as an Acid at Various Temperatures.

Temperature range °C. (1)	Base added (2)	Equivalent weight (3)	Base bound by 1 gm. of protein $1/(3) \times 10^{-4}$ (4)	138/(4) (5)	Whole numbers ascribed to (5) (6)
		gm.			
60–85	Monovalent	3700	27.0	5.1	5.0
21–37	" (25)	2100	47.5	2.9	3.0
60–88	$Ca(OH)_2$	1400	71.5	1.95	2.0
23–25	Paracasein-NaOH	1450	69.0	2.0	2.0
18–25	Maximum base-binding ("unmodified") NaOH (18)	725	138.0	1.0	1.0
" "	Maximum base-binding ("Nach Hammarsten") NaOH (18)	545	183.0	3/4	

1 gm. of this modification of casein binds one-half of the amount necessary to saturate an "unmodified" casein. The system casein-$Ca(OH)_2$ from 60° to 85°C. conforms to the last relation.

Up to the present we have treated the system casein-$Ca(OH)_2$ as being identical with the system casein-monovalent base. The parallel is not strictly correct. A large body of experimental evidence indicates, as we have seen, that casein with $Ca(OH)_2$, at high temperature, forms a rather insoluble salt. If a similar change occurs in the properties of casein in this system, as it does in the system of casein-monovalent base, the relation of the combining weights (Table IX)

to each other indicates that the solubility of casein in $Ca(OH)_2$ becomes independent of the temperature when about two-fifths of the salt formed is soluble in water.

The evidence gathered in this discussion seems to indicate that the base-binding capacity of casein is not only affected discontinuously by the temperature, but that there exists a definite multiple relationship associated with this discontinuous behavior, indicating that this change is of a stoichiometric nature.

SUMMARY.

1. The investigations dealing with the properties of casein as an acid were reviewed.

2. The solubility of uncombined casein in water was measured at 5°C. and found to be 0.70 ± 0.1 mg. of N per 100 gm. of water.

3. Robertson's solubility measurements of casein in bases at various temperatures were recalculated and found to agree well with more recent measurements.

4. By combining the observations of several investigators, as well as the author's measurements of the solubility of casein, in base, at various temperatures, the following conclusions were reached:

(*a*) The solubility of casein in base is affected by the temperature in a discontinuous manner.

(*b*) There exist two ranges of temperature, one, extending from about 21° to 37°C. and the other from about 60° to 85°C. where the solubility of casein in base is practically independent of temperature.

(*c*) From 37° to 60° the equivalent combining weight of casein rises from the value 2100 to about 3700 gm.

5. By comparing the values of base bound by 1 gm. of casein at the two temperature ranges with a constant, the value of base necessary to saturate the same amount of casein, it was found that the latter value is a common multiple of the former values, indicating the stoichiometric nature of the effect of temperature.

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THE EFFECT OF RENNIN UPON CASEIN.

I. The Solubility of Paracasein in Sodium Hydroxide.

By VLADIMIR PERTZOFF.

(From the Department of Physical Chemistry, Laboratories of Physiology, Harvard Medical School, Boston.)

(Accepted for publication, May 13, 1927.)

I.

INTRODUCTION.

When small amounts of rennin, or of any proteolytic enzyme, are added to milk a clot appears. In the course of the clotting a modification occurs in the casein of milk, resulting in the production of a protein that has been called paracasein.[1]

The clotting of milk has been the subject of many investigations, and the explanations of the phenomenon that have been proposed may be classified, on the whole, into two groups. The first of these assumes that the clotting of milk is essentially an incipient proteolysis. The second interprets the reaction in terms of colloidal chemistry and assumes that no chemical modification has taken place, paracasein being only a physical modification of casein. According to this view, the change leading to the clotting of milk involves some colloidal properties of the casein.

Among the exponents of the theories falling under the first group was Hammarsten. As early as 1875 Hammarsten attempted to explain the action of rennin in terms of proteolysis, the casein being split into two parts: one, paracasein, and the other a whey albumin (1) Very similar views were held by Schmidt-Nielson (2) and by Slowtzoff (3). Van Herwerden advanced a modified theory. According to this, the resulting paracasein is made up of two substances, Paracasein *B* and Paracasein *C*. The latter (unlike Paracasein *B*) is not precipitated by acetic acid, and requires more concentrated $(NH_4)_2SO_4$ for its precipitation. It is soluble in

[1] Casein for paracasein, and caseinogen for casein, according to the English classification.

water and gives a strong biuret reaction. After long action a proteose is formed which leads to the further decomposition of the molecule (4).

The analytical identity of casein and paracasein has been the subject of several investigations. Köster (5), Rose and Schulze (6), Raudnitz (7), L. L. Van Slyke and Bosworth (8) and Bleyer and Seidl (9) analyzed paracasein. Harden and Macallum (10) found that the "conversion of caseinogen into casein by enzyme action is accompanied by the cleavage of N, P and Ca. Rennin action produces no soluble N or P. Trypsin splits off both soluble N and P. . . . . The cleavage products are specific for each enzyme and it is to this difference of enzyme action that the variation in behavior of the resulting casein is to be ascribed."

TABLE I.

The Elementary Composition of Pardcasein.

Per cent of the element						Investigator
C	H	N	S	P	Ash	
52.79	6.98	15.84				Köster (1881)
53.94	7.14	15.14	1.01			Rose and Schulze (1885)
				0.66–0.84		Raudnitz (1903)
				0.85–0.87		Kikkoji (1909)
53.50	7.26	15.80	0.87	0.83	0.61	Van Slyke and Bosworth (1913)
53.50	7.26	15.80	0.72	0.71	0.07	Bosworth (1914)
53.47	7.19	15.78	0.72	0.71	0.09	" "
		15.64	0.81	0.83	0.1	Bleyer and Seidl (1922)
		15.70	0.76	0.80	0.085	" " " "
		15.65	0.78	0.79	0.09	" " " "
		15.60				" " " "
		15.61				" " " "
Average....53.45	7.17	15.65	0.81	0.78		
Casein.....53.5–	7.13–	15.62–	0.72–	0.71–		
52.7	6.81	15.80	1.01	0.88		

Geake (11) investigated the elementary compositions of casein and paracasein and found them much alike.

Finally, Bosworth (12), using purified paracasein, found that the composition of paracasein was the same irrespective of the enzyme used to produce it. According to him, casein and paracasein have the same percentage composition. The results of these analyses are given in Table I. On the whole, paracasein does not differ much in elementary composition from casein.

We shall express our results in terms of nitrogen. In some of the calculations, however, it will be necessary to convert these values to

terms of gm. of paracasein. In such calculations we shall use the average value for nitrogen obtained from Table I.

Whether a proteose is split off from the casein molecule by rennin has been a subject of much controversy. For this there seems to have been a twofold reason. Not all clots formed by rennin are alike. Their abundance and physical properties largely depend upon the Ca and H ion concentration of the milk. It follows that when the coagulation occurs, soluble nitrogen can be occluded and absorbed to different extents. Rennin preparations usually contain some proteolytic enzyme, and it has often been contended that rennin is itself a proteolytic enzyme. Therefore, if the proteolytic enzyme is not inactivated in the course of the coagulation by the products of the reaction, it will afterwards produce soluble nitrogen, and even P or S, depending upon the manner in which the complex protein molecule is split.

The study of the nature of the reaction between casein and rennin has been most successfully investigated by the study of the intermediary product of the reaction—the paracasein isolated from milk and subsequently purified. Van Slyke and Bosworth (13), using their methods for the purification of casein and for the purification of paracasein, found that the amount of base just necessary to hold paracasein in solution was almost exactly twice that necessary to dissolve casein. They concluded that:

"the molecule of calcium caseinate containing four equivalents of base is split by rennin into two molecules of paracaseinate, each containing two equivalents of base. Such a paracaseinate is soluble in water, but insoluble in the presence of more than a trace of a soluble calcium salt. A molecule of calcium caseinate containing two equivalents of base is split by rennin into two molecules of paracaseinate, each containing one equivalent of base. Such a paracaseinate is insoluble in water."

Rennin therefore is not regarded by these investigators, strictly speaking, as a coagulating enzyme. Rather they consider the coagulation as a secondary effect, the result of a change in solubility. Further, they believe the action of rennin to be the first step in the proteolysis of casein (14).

Among the exponents of a purely physical point of view of the clotting of milk, Mellanby (15) must be mentioned. He supposed that the enzyme was adsorbed by the casein, thus forming the clot. Alex-

ander (16) suggested that the casein in milk is protected by the lactalbumin; and that the enzyme destroys the lactalbumin, and thus permits the coalescence of the caseinogen particles. Schryver (17), after an extensive investigation of the solubility of casein subjected to various treatments, concluded that in milk:

"the materials necessary for the clot formation pre-exist, but that aggregation formation is prevented by the adsorption of simpler molecules from the system. The conception was formed that a ferment, for which the colloidal substances could act as a substrate, could clear the surface of such substances of adsorbed bodies and thus allow aggregation (clot) formation to take place."

Recently, Wright (18) investigated the racemization curves of casein and paracasein and found them identical. From this fact he concluded that

"rennet does not cause any proteolytic cleavage of the caseinogen molecule, but that coagulation is due to an alteration of the colloid state of the caseinogen by which precipitation in the presence of bivalent metal ions is facilitated."

The present investigation was undertaken on the basis of the simplest chemical hypotheses. In order to know the nature of a reaction, one must know the chemical properties, not only of the reactants, but also of the products. The reactant, casein, is well known because of the extensive investigations of Lacqueur and Sackur, T. B. Robertson, L. L. Van Slyke, E. J. Cohn and their respective coworkers. We propose therefore to study paracasein: its physicochemical properties and behavior. In this we hope to identify paracasein as a chemical substance, in the belief that a more complete knowledge of the reactants and of the end-products of reactions promoted by enzymes may give more reliable information regarding their nature, and perhaps also their mechanism.

II.

The Preparation of Paracasein.

Six different paracasein preparations have been used in this investigation. Of these the protein was in five cases precipitated from milk by active preparations of rennin, and in one by pepsin. The methods used for the purification of the paracasein were much like those already described by L. L. Van Slyke and Baker (19). A modification of their method (20) yielded reproducible results with casein and

with paracasein preparations. Since in the latter case the first precipitation was affected by the enzyme, it was in some cases unnecessary to add any acid. In this only did the method of purification of casein differ from that of paracasein. In one of the paracasein preparations we used a large excess of alkali, in order to study its effect on the protein. No appreciable differences were found between preparations treated with an amount of alkali just necessary to dissolve them and a preparation in which an excess of alkali had been used.

Paracasein I.—An active rennin powder prepared by the Digestive Ferments Company was used in the preparation of Paracasein I. To 7 liters of skimmed milk was added 0.001 gm. of the powder. The milk was then kept for 24 hours at 25°C. under an excess of toluene. After the separation of the clot the precipitate was washed seven times with four times its volume of distilled water. The precipitate was brought into intimate contact with the successive wash waters by means of a motor-driven, glass, screw-shaped stirrer. On each washing the reaction of the the clot appeared to be more acid, until by the seventh washing the pH had become 4.6. This reaction is the optimum for the precipitation of casein (21). The change of the reaction of the precipitate can most easily be accounted for in terms of the Donnan equilibrium; for, since both calcium paracaseinate and uncombined paracasein are relatively insoluble, it may be conceived that transformation of the former into the latter takes place by the diffusion of calcium ions from the casein clot, and their replacement by hydrogen ions. This case is thus comparable with the Donnan theory of hydrolysis through a membrane. It is of interest to note that this phenomenon did not take place with all of our preparations.

After the clot was washed, the paracasein was dissolved by the addition of 0.1 N sodium hydroxide. The reaction of the resulting solution was found to be approximately pH 7.0. The solution was passed through a Sharples centrifuge, and then filtered through filter paper pulp. The paracasein was reprecipitated by means of 0.1 N hydrochloric acid, delivered very slowly from a capillary tip extending well into the solution. The solution was continuously and rapidly stirred. The acid was added until the pH was 4.6, which was found to be the point at which paracasein flocculates the best. Finally, the precipitate was washed with water, redissolved by sodium hydroxide, reprecipitated by hydrochloric acid and again washed until chloride-free. The whole preparation was carried on in the manner that has already been described in detail for casein (20).

Paracasein II.—Pepsin powder prepared by the Parke, Davis and Company was used in the preparation of Paracasein II. To 4 liters of milk was added 0.002 gm. of the powder. The subsequent washing and purification was carried on in the same way as for Paracasein I.

Paracasein III.—Active rennin powder prepared by the Digestive Ferments Company was used in the preparation of Paracasein III. To 3½ liters of milk was added 0.005 gm. of the powder. The clot was washed four times as before, and enough 0.1 N sodium hydroxide was added to bring the solution to pH 9.0. The subsequent purification was carried on in the same way as in the cases of the Paracaseins I and II.

Paracasein IV.—3 liters of milk were warmed to 32°C. Immediately 0.036 gm. of Hansen's salt-free active rennin powder was added to it. The milk was vigorously stirred. After an elapse of 1½ hours, the milk coagulated. It was immediately cooled down to 5°C., and extensively washed with water.

Otherwise the procedures of purification were the same as in Preparations I, II and III.

Paracasein V.—In this preparation, we used the Morgenroth method of coagulation of milk. It is based on the following observation: Milk to which rennin is added and which is kept at a low temperature does not coagulate, although the transformation of casein into paracasein presumably takes place. This milk can be coagulated after a short exposure to high temperature. The usual explanation of this phenomenon is that paracasein, even in the presence of salts, does not coagulate at a low temperature. The coagulation may be brought about by warming the milk.

3 liters of milk were cooled to about 5°C., and 0.01 gm. of Hansen's salt-free rennin preparation was added to it. The milk was placed in a cold room at 5°C., and left undisturbed for 20 hours. No coagulation took place at that temperature. The milk was then warmed to 35°C., which brought about its coagulation in a time slightly less than 30 minutes. After the coagulation took place the milk was cooled to 5°C., and purified by the ways described.

Paracasein VI.—To 3 liters of milk we added, in this preparation, 0.03 gm. of considerably weakened Hansen's salt-free rennin preparation. After an elapse of about 12 hours at about 18°C. the milk coagulated.

The subsequent purification of this preparation of paracasein was carried on in the usual way.

Since, in the preparations of paracasein, an enzyme was added to the systems, they were no longer comparable to systems containing only casein. If, however, the enzyme added were wholly inactivated during the course of the purification, the investigation of paracasein would become comparable with that of casein.

Our pepsin preparation had roughly twice as much enzyme as the rennin preparations, both in respect to coagulative and proteolytic activity. Therefore, Paracasein II had about ten times as much enzyme as Paracasein I. The same relation is true of Paracaseins III and I. But, Paracasein III was also brought to an alkaline reaction in the course of its precipitation, to pH 9.0. It is well known that both pepsin and rennin are very sensitive to alkali, and experiments carried out in this laboratory upon the latter confirm the observation that this enzyme is quickly inactivated even at neutral reactions. We conclude from this that

after purification Paracasein III should contain less enzyme than Paracasein II.

If the property of prepared paracasein were dependent upon the proteolysis produced by rennin or pepsin added, we should expect that Paracasein II, when equilibrated with a certain amount of sodium hydroxide, would produce much more soluble nitrogen than, for example, Paracasein I or III. This was found not to be the case. Preparations of paracaseins differed but slightly from each other, and their solubilities were practically independent of time.

As we shall see from one of the next sections, the solubility of paracasein can be measured both at low and at high temperature. If the solubility is due, even in part, to a proteolytic enzyme, the rise in temperature would increase the solubility. On the contrary, the temperature coefficient of the binding capacity of paracasein with NaOH does not differ at all from the corresponding temperature coefficient of casein.

Therefore, we are inclined to think that in the course of the purification of paracasein the enzyme added was wholly inactivated, and did not interfere appreciably with subsequent measurements.

III.

The Solubility of Paracasein in Water.

Paracasein, purified to the same extent as casein and chloride-free, still contains large amounts of caseose which appear in the solution, after a thorough washing of the suspension. The amount of soluble nitrogen varied slightly from preparation to preparation, and the pH approached 6.5, presumably as a result of the presence of these substances. If we assume that paracasein is soluble in water, the nitrogen found in the filtrate may be represented as being composed of (1) nitrogen arising from the solution of paracasein in water and (2) nitrogen arising from any other soluble substance present in our system. In order to simplify our reasoning, let us consider first a simpler chemical case. Let us assume that we have in a liter of water a large excess of barium sulfate, together with some magnesium sulfate and sodium sulfate, and that we wish to measure the solubility of barium sulfate in water. Let our method for the estimation of barium sulfate be limited

to the measurement of the SO_4 radical only. Should one equilibrate such a mixture and then filter some of it and estimate the gm. of SO_4 present in the filtrate, values for the solubility of barium sulfate, greater than the theoretical solubility, would be obtained. The magnitude of this increment will of course depend upon the amount of more soluble sulfate salts present. An attempt, however, to wash such a system systematically, will result in values ranging closer and closer to the value of the solubility in water of the least soluble substance, barium sulfate in our case. Several criteria for the value of the solubility of barium sulfate in water might be applied. First, it should be independent of the way in which barium sulfate was prepared; in the second place, its solubility should be independent of the length of time the solvent is equilibrated with the saturating body, which should finally dissolve in successive fractions of the same solvent to the same characteritic extent, since a chemically pure substance has always the same physicochemical properties.

The situation with paracasein was very like that with the inorganic analog we have just considered. In our case paracasein, a substance of unknown solubility, was obtained with a mixture of other nitrogen-yielding substances, also of unknown solubility. The method used to obtain a pure substance from this mixture was identical with that described for the purification of barium sulfate.

The experimental procedure was as follows: Samples of Paracaseins I, II and III were placed in three Pyrex bottles. They were diluted with water to about ten times the volumes of the precipitates. The suspensions were stirred by means of a motor-driven, glass, screw-shaped stirrer at about 7°C. The stirring did not change appreciably the state of subdivision of the paracasein precipitate. Only a few hours were required to bring the suspension to the state of equilibrium with the watery phase. The time of stirring was, however, extended to 48 hours and often to more. After each stirring the supernatant liquid was decanted and part of it filtered through a No. 42 Whatman filter. The nitrogens were determined on 50 cc. aliquots by the Kjeldahl method. On the same filtrate a colorimetric pH measurement was taken. Then the bottles were refilled with cold water, and the operation repeated. The results of the experiment are tabulated in Table II.

The results of the experiment show a constancy of the solubility of paracasein in water, when freed from hydrolytic products. Only one measurement, namely, the solubility of Paracasein III on the seventh washing, diverges by a value greater than the experimental error.

We have reason to believe that this preparation became contaminated at this stage of washing. It was probably for this reason that the solubility of this preparation was slightly higher.

There is one more bit of interesting evidence that the system of paracasein and hydrolytic products is very similar to the system of our inorganic analog. Paracasein II, after the first and the third washings (Table II), was stirred for 5 and 10 hours, respectively, and then for 48 and 24 hours. The amount of nitrogen in the liquid phase remained constant. This evidently indicated that the soluble nitrogen

TABLE II.

The Solubility and the Hydrogen Ion Activity of Paracasein in Water.

Time of stirring	No. of washings	Solubility: mg. N in 25 cc. Paracasein preparation			pH Paracasein preparation		
		I	II	III	I	II	III
hrs.							
5	1		1.37			6.5	
53	1		1.37			6.5	
10	2		0.77				
10	3		0.48			6.4	
34	3		0.47				
12	4	0.35	0.33	0.41	6.4	6.4	6.4
48	5	0.14	0.16	0.17	6.4	5.25	6.2
48	6	0.14	0.15	0.20	5.25	5.25	5.25
72	7	0.15	0.17	0.25	5.1	5.2	5.25
48	8		0.15				
144	8	0.16					

did not arise from the activity of a proteolytic enzyme, since then, with time, the amount of protein hydrolyzed should increase. This was not the case. Our systen was a mixture of two or more substances differing in their solubility in water.

The average of all the determinations from the fifth down to the eighth washing gave the value of 0.17 ± 0.03 mg. of nitrogen in 25 cc. The solubility of paracasein per 1000 gm. of water is therefore equal to 6.8 ± 1.2 mg. of nitrogen, at about 7°C.

The pH of a purified paracasein is most probably 5.2. It is more alkaline than the pH at which paracasein is best precipitated, which is in the neighborhood of pH 4.6.

In an investigation of the effect of temperature upon the solubility of casein in water (22), we found that this protein dissolves to an extent of 7.0 ± 1.0 mg. protein N in 1000 gm. of water at 5°C. Comparing this solubility with the one obtained for paracasein, it is evident

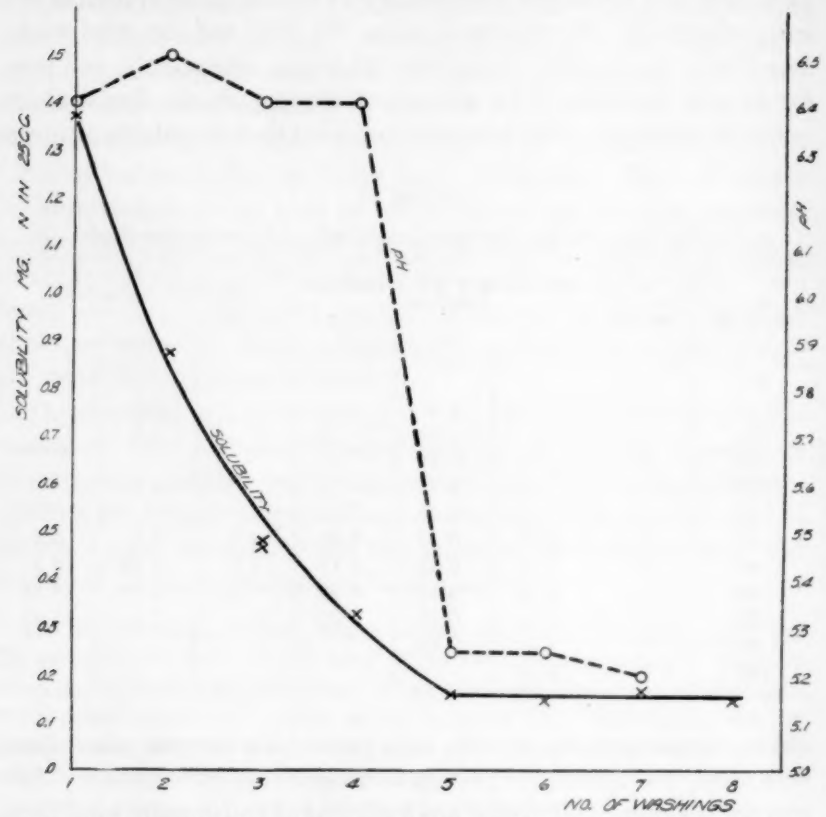

FIG. 1. The purification of paracasein from hydrolytic products and its solubility in water in an uncombined state.

that the solubilities of these two proteins are identical at practically the same temperature. We may conclude that paracasein, at low temperature, dissolves in water approximately to the same extent as casein.

IV.

The Solubility of Paracasein in Sodium Hydroxide.

Similarly to casein, when sodium hydroxide is added to paracasein, this protein forms a sodium compound which is soluble in water. By

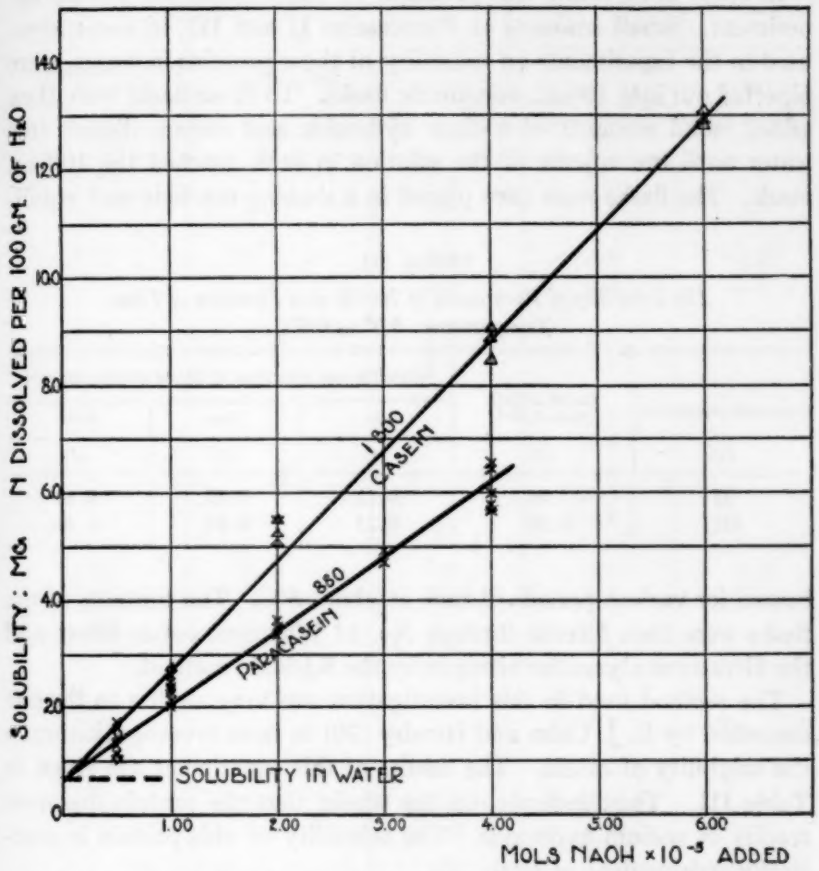

FIG. 2. The solubility of paracasein in NaOH at 5°C. as compared with the solubility of casein at the same temperature (22).

estimating the amount of paracasein dissolved by a given amount of sodium hydroxide, we can measure the combining capacity of this protein as an acid, provided all the base is bound by the protein in solution.

Before undertaking the actual measurements of the solubility of the protein, it seems to be advisable to determine the time in which this system will come to equilibrium. A sufficient criterion for this state for an isothermal, isopiestic system is the independence from time of the amount of protein dissolved by a given amount of base.

In order to elucidate this problem, we undertook the following experiment. Small amounts of Paracaseins II and III, in suspension, used in the experiments on solubility of these proteins in water, were pipetted out into 100 cc. volumetric flasks. To these flasks were then added small amounts of sodium hydroxide and carbon dioxide-free water until the volume of the solution in each reached the 100 cc. mark. The flasks were then placed in a shaking machine and equili-

TABLE III.

The Solubility of Paracasein in NaOH as a Function of Time.
Temperature: 5.0° ± 0.5°C.

Paracasein preparation	NaOH added: mols × 10^{-4}	Solubility: mg. N in 20 cc. of filtrate after an elapse of		
		1 hr.	3 hrs.	6 hrs.
(1)	(2)	(3)	(4)	(5)
II	1.00	0.52	0.52	0.54
III	0.50	0.23	0.23	0.24

brated for various periods of time at about 5°C. The contents of the flasks were then filtered through No. 42 Whatman paper filters and the filtrates analyzed for nitrogen by the Kjeldahl method.

The method used in this investigation was very similar to the one described by E. J. Cohn and Hendry (20) in their investigation upon the solubility of casein. The results of this experiment are given in Table III. They indicate, on the whole, that the protein dissolves readily in sodium hydroxide. The solubility of this protein is practically independent of time.

The investigation of the solubility of paracaseins in sodium hydroxide was then undertaken. The method for the determination of soluble nitrogen was identical to that one already described. The time of equilibration was varied from 2 to 24 hours. The temperature at which the experiments were carried out was 5.0° ± 0.5° C. and 23.0° ± 2°C.

The amount of the saturating body was varied within large limits without any appreciable effect upon the solubility of paracasein.

TABLE IV.

The Solubility of Paracasein in Sodium Hydroxide at 5.0° ± 0.5°C.

NaOH added: mols × 10^{-4}	Experiment No.	Paracasein preparation		
		I	II	III
		Mg. N dissolved per 100 gm. of water		
(1)	(2)	(3)	(4)	(5)
0.50	4 5 1	1.4 1.4	1.6 1.7	1.1 1.3
1.00	2 1 2	2.4 2.3	2.6 2.6	2.0 2.1
	4 2	2.3 2.4	2.6 2.7	
	3		2.4 2.4	
	5		2.4 2.6	
2.00	3 2 2	3.4 3.5	3.5 3.6	3.5
	4 5	3.4	3.4 3.4	
3.00	5		4.7 4.9	
4.00	3 5	5.65 5.75	6.45 6.55	
	4	5.65 6.00		

Paracasein is much like casein in this respect: the solubility with sodium hydroxide is independent of the amount of the protein in the precipitate, and is solely determined by the amount of the base added.

The results of these investigations are recorded in Tables IV and V, and Fig. 2.

The solubility of paracasein at 5°C. in NaOH (Fig. 2), unlike its solubility in water, is distinctly different from that of casein. An equal amount of base carries less paracasein into solution than it does casein. About 810 gm. of paracasein are carried into solution by 1 mol of NaOH.

TABLE V.

The Solubility of Paracasein in Sodium Hydroxide at 23° ± 2°C.

Paracasein preparation (1)	Amount of NaOH added: mols × 10^{-5} (2)	Solubility: mg. N dissolved per 100 gm. of water (3)	Equivalent weight: gm. of paracasein (4)
I	2.00	3.9	
	6.00	12.9	1450
II	0.50	(1.8)	
	1.00	3.3	
	5.00	11.9	1400
II	2.00	4.9	
	6.00	13.5	1400
IV	2.00	3.65	
	6.00	13.2	1550
V	2.00	5.35	
	4.00	10.1	1500
Average.....	.....	.....	1450
VI	2.00	5.17	
	4.00	10.55	1700

We have repeated these measurements at a temperature of 23° ± 2°C. In these investigations we have included Paracasein Preparations I and II, as well as IV, V and VI.

The results of these measurements are recorded in Table V. As in the measurements at 5°C., paracasein is distinctly different from casein at 23° ± 2°C.: its average equivalent is 1450, while casein in the

corresponding range of temperature had the equivalent weight of 2100 ± 100 gm. (20).

The equivalent weight of Paracasein Preparation VI, which was

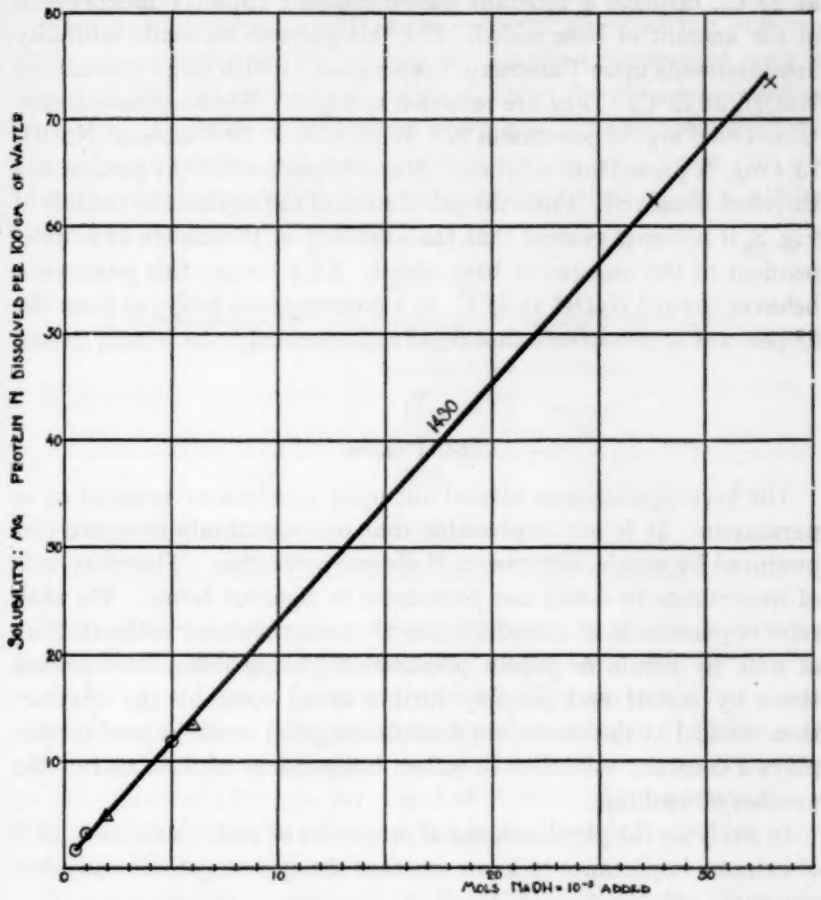

FIG. 3. The solubility of Paracasein Preparation II in small and large amounts of NaOH at 23° ± 2°C.

prepared by the use of a considerably weakened rennin preparation, is outside our experimental error. The equivalent weight of this preparation is about 1700 (Table V). We shall consider this paracasein preparation in the following communication.

In all of these investigations, we have confined ourselves to estimates of the solubility of paracasein with small amounts of sodium hydroxide. It is of interest whether paracasein, like casein (20) at 25°C., displays a constant base-combining capacity independent of the amount of base added. For this purpose we made solubility measurements upon Paracasein Preparation II with large amounts of NaOH, at 25°C. They are recorded in Fig. 3. The suspensions contained 86.0 mg. of paracasein N. With 32.9×10^{-5} mols of NaOH, 73.4 mg. N passed into solution. About 85 per cent of the protein was therefore dissolved. From the calculation of the equivalent weights of Fig. 3, it becomes evident that the solubility of Paracasein II is independent of the amount of base added. Like casein, this paracasein behaves toward NaOH at 25°C. as a homogeneous body; at least the 85 per cent of precipitate that dissolved appeared to be homogeneous.

V.

CONCLUSIONS.

The investigation was carried out upon a substance referred to as paracasein. It is not improbable that one can obtain from the clot produced by rennin, substances of diverse properties. Therefore, it is of importance to define our paracasein in rigorous terms. We shall refer to paracasein as a modification of casein produced in the clotting of milk by rennin or pepsin preparations, being redissolved several times by NaOH and possibly further acted upon by the enzyme; then washed at the maximum flocculation point until the product displays a constant solubility in water, independent of time and of the number of washings.

In studying the physicochemical properties of such a substance, it is of extreme importance to know whether the system contains any free enzymes.

The absence of the proteolytic enzyme from our parcasein preparation was concluded from the following observations: (1) The amount of enzyme originally added to the milk has no bearing upon the solubility of the product in water or NaOH. (2) The time factor has no effect upon the solubility of paracasein in water. (3) The solubility with sodium hydroxide is practically independent of time. (4) The

temperature coefficients of the solubilities of casein and paracasein in sodium hydroxide are identical (Table VI). To our mind, these criteria are sufficient to conclude that no active proteolytic enzyme was present in our purified paracasein preparations.

Paracasein dissolves in water to an extent of about 7.0 mg. of N per 1000 gm. of water at 7°C., which within the experimental error is identical with the solubility of casein at about the same temperature, since the properties of paracasein as an acid are distinctly different from those of casein, one may conclude that, in some cases, one can change the acid properties of a protein without affecting its solubility in water.

TABLE VI.

A Comparison of the Acid Properties of Paracasein and Casein.

Solubility range: mols NaOH × 10^{-4}	Temperature, °C.	Equivalent weight: gm. of protein		Ratio (3)/(4)
		Casein	Paracasein	
(1)	(2)	(3)	(4)	(5)
0–6.00	5.0	1300		1.48
0–4.00	5.0		880	
Any	21–37	2100		1.45
"	21–25		1450	

As we have already pointed out, paracasein is distinctly different from casein in its capacity to bind base, both at 5° and at 23° ± 2°C.

At 5°C., paracasein, in most of the solubility regions investigated, dissolves at a rate of 810 gm. of the protein per 1 mol of NaOH. At the corresponding temperature and solubility range, casein dissolves to the extent of 1300 gm. per 1 mol of NaOH. At the temperature level of 21°–25°C., the corresponding figures are 1450 gm. for paracasein and 2100 gm. for casein.

This information is compiled in Table VI. In column (5) of this table is given the ratio of the equivalent weight of casein to the equivalent weight of paracasein. The relation seems to yield a constant, which indicates that the temperature coefficients of the solubility of paracasein and casein in NaOH are identical.

As the calculation of Table VI shows, paracasein is nearly 1.5 times

more acid than casein, the capacities of casein and paracasein to bind base standing to one another as the whole numbers 2 and 3 (22).

The fact that in using varying amounts of the enzyme one obtains practically identical parcaseins indicates that most of the paracaseins obtained in this investigation are final products of a reaction promoted or brought about by rennin or pepsin.

The information gathered in this research substantiates the conclusion reached by several investigators, that the transformation of casein into paracasein is of a chemical nature.

VI.

SUMMARY.

1. The preparation and purification of paracasein was described and certain criteria for the absence of free enzyme provided for.

2. The solubility of purified paracasein in water at low temperature was studied, and found practically identical with the solubility of casein.

3. The capacity of paracasein to bind base was investigated by means of its solubility in NaOH at 5° and at 23° ± 2°C., and found to be distinctly different from that of casein.

4. At these two temperature levels paracasein had a 1.5 greater capacity to bind base than casein. The equivalent combining weights of paracasein and casein were found to stand each to the other, ap-approximately, as 2 to 3.

5. This relationship suggested that the temperature coefficients of the solubility of paracasein and casein in NaOH are identical.

6. This evidence indicates that paracasein is a modification of casein, distinguishable by physicochemical means.

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